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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: J. Troy WEEKS et al.

Title: Refined Plant Transformation

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CONTINUING PATENT APPLICATION
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Sir:

Transmitted herewith for filing under 37 C.F.R. § 1.53(b) is a:

Continuation Division Continuation-In-Part (CIP)

of the above-identified copending prior application in which no patenting, abandonment, or termination of proceedings has occurred. Priority to the above-identified prior application is hereby claimed under 35 U.S.C. § 120 for this continuing application. The entire disclosure of the above-identified prior application is considered as being part of the disclosure of the accompanying continuing application and is hereby incorporated by reference therein.

Enclosed are:

- Application Data Sheet (37 CFR 1.76).
- Specification, Claim(s), and Abstract (114 pages).
- Formal drawings (4 sheets, Figures 1-4).
- Information Disclosure Statement.
- Form PTO/SB/08 with copies of three (3) listed reference(s).

The filing fee is calculated below:

	Claims as Filed	Included in Basic Fee	Extra Claims	Rate	Fee Totals
Basic Fee				\$750.00	\$750.00
Total Claims:	53	- 20	= 33	x \$18.00	\$594.00
Independents:	6	- 3	= 3	x \$84.00	\$252.00
If any Multiple Dependent Claim(s) present:			+ \$280.00	= \$0.00	
Surcharge under 37 CFR 1.16(e) for late filing of Executed Declaration			+ \$130.00	= \$130.00	
			SUBTOTAL:	=	\$1,726.00
[] Small Entity Fees Apply (subtract ½ of above):				=	\$0.00
			TOTAL FILING FEE:	=	\$1,726.00

[X] The required filing fees are not enclosed but will be submitted in response to the Notice to File Missing Parts of Application.

[X] The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

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U.S. PATENT APPLICATION

for

Refined Plant Transformation

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REFINED PLANT TRANSFORMATION

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0001] This application is a Continuation-In-Part of U.S. application serial number 10/392,301, filed 03/20/2003, entitled, "Refined Transformation," which claims priority to U.S. provisional applications 60/365,527, filed 03/20/2002, and 60/377,597, filed 05/06/2002, which are all incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] The ability to transform plants by integrating and expressing desirable polynucleotides in plant cells makes it possible to efficiently introduce agronomic and quality traits into a variety of plant species. Transgenic plants that are produced by current transformation methods, however, require extensive tissue culture manipulations, which are time consuming and species specific. Furthermore, such methods do not only integrate the desirable polynucleotide(s) into a plant's genome, but also additional and superfluous nucleic acids. When making a genetically engineered food, the superfluous nucleic acids may be undesirable because they are from non-food sources, such as viruses and bacteria and are, therefore, undesirable.

[0003] Existing plant transformation methods rely on the use of *Agrobacterium* for DNA transfer. These methods typically comprise (1) preparing tissue explants, (2) infecting explants with at least one disarmed *Agrobacterium* strain, (3) culturing and selecting the transformed plant cells on tissue culture media, and (4) inducing proliferation and subsequent regeneration to generate whole plants. Examples of these methods are described in U.S. Patent Nos. 5,591,616, 6,051,757, 5,164,310, and 5,693,512, and EP 0 672 752 A1, which are incorporated herein by reference. However, explant preparation is a laborious process that requires extensive

resources, especially for many monocotyledonous plant species including maize, wheat, barley, and oats.

[0004] Furthermore, the subsequent process of proliferation and regeneration is also very laborious, taking at least 12 months to develop a primary transformed plant. Since different plants require different concentrations of salts, minerals, and hormones, including auxins and cytokinins, for proliferation and regeneration, the applicability of typical transformation methods is limited to one species or only a few cultivars of one species.

[0005] Even by optimizing cultivar-specific transformation methods, successful transformation has been accomplished for only a very few cultivars of important crop species, such as for the maize inbred lines H99, Oh43, and B73, the spring wheat variety Bobwhite, and the cotton cultivar Coker 312. The introduction of foreign DNA into elite germplasm often requires the transformation of inferior cultivars followed by conventional multi-year breeding programs to introgress the DNA into the desired material.

[0006] Tissue culture manipulations can be avoided by either vacuum infiltrating plants with an *Agrobacterium* suspension or emerging such plants in suspensions that also contain approximately 0.05% Silwet L-77 (Bechtold et al., *Acad Sci Paris Life Sci* 316: 1194-1199, 1993; Clough & Bent, *Plant J* 16: 735-743, 1998). However, this method is only applicable to the model plant systems *Arabidopsis thaliana*, *Arabidopsis lasiocarpa*, and *Raphanus sativus*. Transgenic plants can also be obtained for a fourth plant species, *Medicago trunculata*, by vacuum infiltrating seedling with *Agrobacterium* suspensions.

[0007] Such *in planta* transformation systems are of limited utility, however, and not applicable to commercially relevant crop plants. Efforts to broaden such applicability to encompass a larger variety of crops have failed because of the inaccessibility of those crops to *Agrobacterium*-mediated

transformation, and/or the resultant, detrimental physiological responses, such as flower abscission and *Agrobacterium*-induced necrosis.

[0008] Other plant transformation methods include those described in EP 1198985 , and US patent application 03/0135891, which immerse germinated seeds into *Agrobacterium* solutions and then select a transformed seed. For instance the '985 patent describes the transformation of rice seedlings by immersing 5-day old seedlings in an *Agrobacterium* suspension, incubating them for 2 weeks on a proliferation and selection medium, and then allowing for a 4-week regeneration phase. Alternatively, 3 to 4-week old seedlings that are cut in the shoot apex region, including the apical meristem, are used to transform pine. The treated seedlings are immersed in *Agrobacterium*, incubated for 2 to weeks on selection medium, and transferred to soil. However, the transformation efficiency of such methods are reported to be extremely low. Their utility, therefore, is very limited.

[0009] Alternative transformation systems include direct DNA delivery systems like particle bombardment (U.S. Patent No. 4,945,050), polyethylene glycol treatment (U.S. Patent No. 6,143,949), microinjection (U.S. Patent No. 4,743,548), whiskers (U.S. Patent No. 5,302,523), and electroporation (U.S. Patent No. 5,284,253). Whereas DNA transfer mediated by *Agrobacterium* is often limited to one to three copies of foreign DNA, direct DNA delivery systems usually result in the transfer of many more copies, which may integrate randomly throughout the plant genome. The unnecessary abundance of insertions is undesirable and may negatively affect the plant genome's integrity.

[0010] Sonication was shown to greatly enhance the efficiency of both *Agrobacterium*-mediated transformation and direct DNA delivery (U.S. Patent 5,693,512). The ultrasound vibrations are believed to disrupt cell walls and thereby facilitate foreign DNA transfer. Sonication reduces the viability of tissue explants, and any increase in transformation frequency may be compromised by an increase in non-viable or dying plants.

[0011] These, as well as more conventional transformation methods, introduce a variety of viral and bacterial genetic elements into plant cells. At least four different genetic elements, derived from bacteria, are typically used to transform plants (During, *Transgenic Research* 3: 138-40, 1994). Such elements include regulatory sequences such as promoters and terminators to promote appropriate transgene expression in plants. An example of a frequently used foreign promoter is the 35S "super" promoter of Cauliflower Mosaic Virus (CaMV), which is able to not only induce high levels of expression of the transgenes but also enhance the expression of native genes in its vicinity (Weigel et al., *Plant Physiol.*, 122: 1003-13, 2000).

[0012] Other strong viral promoters include those from rice tungro bacilliform virus, maize streak virus, cassava vein virus, mirabilis virus, peanut chlorotic streak caulimovirus, figwort mosaic virus and chlorella virus. Other frequently used promoters are derived from bacterial species and include the promoters of the nopaline synthase and octopine synthase gene. Only a few strong and constitutive promoters are derived from food sources. Examples of such promoters are the promoters of the maize Ubiquitin-1 gene (U.S. Patent 6,054,574; and WO 01/94394), the sugarcane Ubiquitin-4 gene (U.S. Patent application 02/0046415), and the potato Ubiquitin-7 gene (Garbarino et al., U.S. Patent 6,448,391 B1, 2002). The applicability of most other plant promoters is limited because of low activity, tissue specificity, and/or poor developmental regulation. Typical terminators are those associated with the nopaline synthase and octopine synthase genes from *Agrobacterium*.

[0013] Also required for transformation is the *Agrobacterium*-derived transfer DNA, i.e., the T-DNA, which transfers desired polynucleotide(s) from *Agrobacterium* into plant cell genomes. Thus, transgenic plants of the conventional art contain much superfluous foreign DNA. Furthermore, the infidelity of DNA transfer can result in co-integration of bacterial plasmid sequences that are adjacent to the T-DNA. In fact, about 75% of transformation events in plants such as tomato, tobacco, and potato may

contain such superfluous plasmid backbone DNA (Kononov et al., *Plant J.* 11: 945-57, 1997). The presence of backbone sequences is undesirable because they contain bacterial origins of replication and/or encode for antibiotic resistance genes.

[0014] Thus, there is a need for accelerated and species-independent methods for transferring and expressing desired polynucleotides into plant cells and genomes. There is also a need to limit the co-transfer of superfluous, undesirable DNA, if the target plant is a food crop. Such methods are provided herein. To optimize DNA transfer from *Agrobacterium* to individual plant cell nuclei, plant tissues such as seedlings are agitated in an *Agrobacterium* suspension. To optimize the subsequent integration of the transferred DNAs into the genome of plant cell nuclei, the plant tissues are exposed to chemicals that induce double strand breaks. Vectors are used that are designed to limit the transfer of undesirable DNA.

SUMMARY OF THE INVENTION

[0015] According to the present invention, a method ("method 1") for producing a transgenic plant is provided. The method comprises (a) agitating a solution comprising a germinating plant seedling, or explant thereof, and at least one *Agrobacterium* strain that harbors a plasmid vector carrying a desired polynucleotide; (b) cultivating the seedling to produce a plant; and (c) screening the plant to determine if the desired polynucleotide is integrated into the genome of at least one cell of the plant, wherein the plant is stably transformed, and wherein the step of agitating the solution does not comprise sonication.

[0016] In one preferred embodiment the germinating plant seedling is from a monocotyledenous plant. In another embodiment, the monocotyledenous plant is selected from the group consisting of turfgrass, wheat, maize, rice, oat, barley, orchid, iris, lily, onion, and sorghum. In another embodiment, the turfgrass is selected from the group consisting of *Agrostis*

spp. (bentgrass species including colonial bentgrass and creeping bentgrasses), *Poa pratensis* (kentucky bluegrass), *Lolium spp.* (ryegrass species including annual ryegrass and perennial ryegrass), *Festuca arundinacea* (tall fescue) *Festuca rubra commutata* (fine fescue), *Cynodon dactylon* (common bermudagrass); *Pennisetum clandestinum* (kikuyugrass), *Stenotaphrum secundatum* (st. augustinegrass), *Zoysia japonica* (zoysiagrass), and *Dichondra micrantha*.

[0017] In another preferred embodiment, the germinating plant seedling is from a dicotyledenous plant. In one embodiment, the dicotyledenous plant is selected from the group consisting of cotton, tobacco, *Arabidopsis*, tomato, potato, sugar beet, broccoli, cassava, sweet potato, pepper, poinsettia, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, geranium, and cactus.

[0018] In another embodiment, the expression of the desired polynucleotide in the stably transformed plant confers a trait to the plant selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity, and production of novel proteins or peptides.

[0019] In a preferred embodiment, the desired polynucleotide of the present invention is selected from the group consisting of a gene or part thereof, the 5'-untranslated region of the gene, the 3'-untranslated region of the gene, the leader sequence associated with the gene, or the trailer sequence associated with the gene.

[0020] In a preferred embodiment, the gene encodes a protein that is selected from the group consisting of an antifungal, a nutritional peptide or protein, a transcription factor, a receptor that binds to pathogen-derived ligands, a hemoglobin, an oxidase, an enzyme of the lignin biosynthesis pathway, an enzyme of industrial value, or an antigen.. Preferably, the desired polynucleotide is operably linked to a promoter and a terminator.

[0021] In a preferred embodiment, the sequences of the promoter and the terminator naturally occur in the genome of plants, or are isolated from human food sources.

[0022] According to the method, the vector comprises (a) a T-DNA or a P-DNA that comprises (i) the desired polynucleotide, and (ii) a selectable marker gene operably linked to a terminator that is not naturally expressed in plants; and (b) a backbone integration marker gene, wherein the desired polynucleotide and the selectable marker gene are positioned between the border sequences of the T-DNA or between the border-like sequences of the P-DNA, and wherein the backbone integration marker gene is not positioned within the T-DNA or within the P-DNA.

[0023] In one embodiment, the desired polynucleotide in the vector is operably linked to a promoter and a terminator.

[0024] In another embodiment, the backbone integration marker gene is operably linked to a promoter and a terminator. In one embodiment, the backbone integration marker is a cytokinin gene. In yet another embodiment, the cytokinin gene is *IPT*, and the plant is a dicotyledon plant. In another embodiment, the backbone integration marker is *PGA22*, *TZS*, *HOC1*, *CKI1*, or *ESR1*.

[0025] In yet another embodiment, the border-like sequences of the P-DNA range in size from 20 to 100 bp and share between 52% and 96% sequence identity with a T-DNA border sequence from *Agrobacterium tumefaciens*.

[0026] In another embodiment, expression of the selectable marker gene confers fertilizer tolerance to the transgenic plant and progeny thereof.

[0027] In another embodiment, the selectable marker gene that confers fertilizer tolerance is a selectable marker gene that confers resistance to cyanamide.

[0028] In another embodiment, the selectable marker gene that confers resistance to cyanamide is selected from the group consisting of *CAH* and *CAH* homologs derived from certain cyanamide tolerant soil fungi including *Aspergillus*, *Penicillium*, and *Cladosporium*. In another embodiment, the selectable marker gene is operably linked to a yeast ADH terminator. In another embodiment, the selectable marker gene is an antibiotic resistance gene. In yet another embodiment, the antibiotic resistance gene is selected from the group of genes encoding hygromycin phosphotransferase, neomycin phosphotransferase, streptomycin phosphotransferase, and bleomycin-binding protein. In another embodiment, the selectable marker gene is a herbicide resistance gene. In another embodiment, the herbicide resistance gene is selected from the group of genes encoding 5-enolpyruvylshikimate-3-phosphate synthase, glyphosate oxidoreductase, glyphosate-N-acetyltransferase, and phosphinothricin acetyl transferase.

[0029] In a preferred embodiment, the step of agitating the solution is accomplished by vortexing. In another embodiment, the solution is vortexed from about 60 seconds to several hours. In yet another embodiment, the solution is vortexed for about 5 minutes to about 30 minutes.

[0030] In one other embodiment, the step of cultivating the seedling to produce a transgenic plant comprises transferring the *Agrobacterium*-transformed seedling to soil, and exposing the transformed seedling to conditions that promote growth.

[0031] In another embodiment, the step of cultivating the seedling to produce transgenic plants comprises cultivating the *Agrobacterium*-

transformed seedling in or on tissue culture medium prior to transferring the transformed seedling to soil, and exposing the transformed seedling to conditions that promote growth.

[0032] The method further comprises (i) producing a callus from the transformed seedling cultivated on tissue culture medium; and (ii) inducing shoot and root formation from the callus, prior to transferring to soil. In this case, the transformation vector may comprises (a) a T-DNA or a P-DNA that comprises (i) the desired polynucleotide, and (ii) a selectable marker gene operably linked to a terminator that is not naturally expressed in plants; and (b) a backbone integration marker gene, wherein the desired polynucleotide and the selectable marker gene are positioned between the border sequences of the T-DNA or between the border-like sequences of the P-DNA, and wherein the backbone integration marker gene is not positioned within the T-DNA or within the P-DNA.

[0033] Furthermore, in one embodiment, the step of producing a callus from the transformed seedling comprises (i) transferring the transformed seedling to tissue culture media that contains auxin and cyanamide; (ii) selecting fertilizer-tolerant calli; (iii) inducing shoot and root formation from the calli; and (iv) transferring calli with shoots and roots to soil and exposing the calli to conditions that promote growth of the transgenic plants from the calli.

[0034] According to method 1, the transformed plant seedling is grown to maturity, crossed to a non-transformed plant and the desired polynucleotide transmitted to at least one progeny plant.

[0035] In another embodiment, the transformed plant seedling is grown to maturity, selfed, and the desired polynucleotide transmitted to progeny.

[0036] In another aspect of the invention a transformation vector is provided. In one embodiment, the vector can be maintained in

Agrobacterium, and comprises: (a) a T-DNA or a P-DNA that comprises (i) a desired polynucleotide, and (ii) a selectable marker gene that is operably linked to a terminator not naturally expressed in plants, and (b) a backbone integration marker gene, wherein the desired polynucleotide and the selectable marker gene are positioned between the border sequences of the T-DNA or between the border-like sequences of the P-DNA, and wherein the backbone integration marker gene is not positioned within the T-DNA or within the P-DNA. In another embodiment, the desired polynucleotide is operably linked to a promoter and a terminator.

[0037] In another preferred embodiment, the backbone integration marker gene is operably linked to a promoter and a terminator.

[0038] In another embodiment, the backbone integration marker gene is operably linked to a promoter and a terminator. In one embodiment, the backbone integration marker is a cytokinin gene. In yet another embodiment, the cytokinin gene is *IPT*, and the plant is a dicotyledon plant. In another embodiment, the backbone integration marker is *PGA22*, *TZS*, *HOC1*, *CKI1*, or *ESR1*.

[0039] In yet another embodiment, the border-like sequences of the P-DNA range in size from 20 to 100 bp and share between 52% and 96% sequence identity with a T-DNA border sequence from *Agrobacterium tumefaciens*.

[0040] In another embodiment, expression of the selectable marker gene confers fertilizer tolerance to the transgenic plant and progeny thereof.

[0041] In another embodiment, the selectable marker gene that confers fertilizer tolerance is a selectable marker gene that confers resistance to cyanamide.

[0042] In another embodiment, the selectable marker gene that confers resistance to cyanamide is selected from the group consisting of *CAH*

or CAH homologs derived from certain cyanamide tolerant soil fungi including *Aspergillus*, *Penicillium*, and *Cladosporium*. In another embodiment, the selectable marker gene is operably linked to a yeast ADH terminator. In another embodiment, the selectable marker gene is an antibiotic resistance gene. In yet another embodiment, the antibiotic resistance gene is selected from the group of genes encoding hygromycin phosphotransferase, neomycin phosphotransferase, streptomycin phosphotransferase, and bleomycin-binding protein.. In another embodiment, the selectable marker gene is a herbicide resistance gene. In another embodiment, the herbicide resistance gene is selected from the group of genes encoding 5-enolpyruvylshikimate-3-phosphate synthase, glyphosate oxidoreductase, glyphosate-N-acetyltransferase, and phosphinothricin acetyl transferase.

[0043] In another embodiment, the promoter and the terminator naturally occur in plants. In another embodiment, the desired polynucleotide comprises a gene derived from an edible food source.

[0044] In one embodiment, expression of the desired polynucleotide in the transformation vector confers a trait to plants that comprise the desired polynucleotide in their genomes, wherein the trait is selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, and improved flower longevity.

[0045] In another aspect of the present invention, a method ("method 2") for producing a transgenic plant, comprising: (A) infecting plant tissue with an *Agrobacterium* transformation vector that comprises (i) a T-DNA or a P-DNA that comprises (a) the desired polynucleotide, and (b) a selectable

marker gene operably linked to a terminator that is not naturally expressed in plants; and (ii) a backbone integration marker gene, wherein the desired polynucleotide and the selectable marker gene are positioned between the border sequences of the T-DNA or between the border-like sequences of the P-DNA, and wherein the backbone integration marker gene is not positioned within the T-DNA or within the P-DNA; (B) cultivating the seedling to produce plants; and (C) screening the plants for stable integration of the desired polynucleotide.

[0046] In one embodiment, the plant tissue is a germinating plant seedling. In another embodiment, the desired polynucleotide is operably linked to a promoter and a terminator. In another embodiment, the backbone integration marker gene is operably linked to a promoter and a terminator. In one embodiment, the backbone integration marker is a cytokinin gene. In yet another embodiment, the cytokinin gene is *IPT*, and the plant is a dicotyledon plant. In another embodiment, the backbone integration marker is *PGA22*, *TZS*, *HOC1*, *CKI1*, or *ESR1*..

[0047] In yet another embodiment, the border-like sequences of the P-DNA range in size from 20 to 100 bp and share between 52% and 96% sequence identity with a T-DNA border sequence from *Agrobacterium tumefaciens*.

[0048] In another embodiment, expression of the selectable marker gene confers fertilizer tolerance to the transgenic plant and progeny thereof.

[0049] In another embodiment, the selectable marker gene that confers fertilizer tolerance is a selectable marker gene that confers resistance to cyanamide.

[0050] In another embodiment, the selectable marker gene that confers resistance to cyanamide is selected from the group consisting of *CAH* and functional *CAH* homologs. In another embodiment, the selectable marker gene is operably linked to a yeast ADH terminator. In another embodiment,

the selectable marker gene is an antibiotic resistance gene. In yet another embodiment, the antibiotic resistance gene is selected from the group of genes encoding hygromycin phosphotransferase, neomycin phosphotransferase, streptomycin phosphotransferase, and bleomycin-binding protein.. In another embodiment, the selectable marker gene is a herbicide resistance gene. In another embodiment, the herbicide resistance gene is selected from the group of genes encoding 5-enolpyruvylshikimate-3-phosphate synthase, glyphosate oxidoreductase, glyphosate-N-acetyltransferase, and phosphinothricin acetyl transferase.

[0051] In another embodiment, the step of cultivating the seedling comprises (i) transferring the *Agrobacterium*-transformed seedling to soil and exposing the transformed seedling to conditions that promote growth.

[0052] In another embodiment, the step of screening the plants for stable integration of the desired polynucleotide comprises (i) exposing the plants to a screening solution containing a substance that only plants that express the selectable marker gene are tolerant to; (ii) growing the plants to maturity and allowing the plants to produce T1 seedlings; (iii) transferring the T1 seedlings to soil; and (iv) exposing the seedlings to the screening solution.

[0053] In another embodiment, the step of infecting the germinating plant seedling comprises submerging the seedling into a solution comprising an *Agrobacterium* strain that contains the *Agrobacterium* transformation vector; and (b) vortexing the solution.

[0054] In another embodiment, the selectable marker gene is operably linked to a yeast ADH terminator.

[0055] In another embodiment, the promoter and the terminator naturally occur in plants.

[0056] In another embodiment, the desired polynucleotide is a plant gene.

[0057] In another embodiment, expression of the desired polynucleotide in method 2 confers a trait to plants that comprise the desired polynucleotide in their genomes, wherein the trait is selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, and improved flower longevity.

[0058] In one embodiment, the substance contained in the screening solution is hydrogen cyanamide.

[0059] In another aspect, a method ("method 3") is provided for modifying the expression of a functional gene in a plant cell comprising:

[0060] (a) constructing a first T-DNA or P-DNA that comprises a desired polynucleotide that is capable of modifying the expression of a functional gene in a plant cell;

[0061] (b) constructing a second T-DNA or P-DNA that comprises a selectable marker gene operably linked to a promoter and terminator, wherein the terminator does not naturally occur in plants;

[0062] (c) exposing germinating plant seedlings to one or more *Agrobacterium* strains that contain the first T-DNA or P-DNA and the second T-DNA or P-DNA;

[0063] (d) selecting only those transformed seedlings that transiently express the selectable marker gene; and

[0064] (e) selecting from the seedlings of (d), a seedling that comprises in its genome the desired polynucleotide but not the selectable marker; wherein expression of the desired polynucleotide in the seedling of (e) modifies the expression of a functional gene in a plant cell in the seedling.

[0065] In one preferred embodiment the germinating plant seedling is from a monocotyledenous plant. In another embodiment, the monocotyledenous plant is selected from the group consisting of turfgrass, wheat, maize, rice, oat, wheat, barley, orchid, iris, lily, onion, and sorghum. In another embodiment, the turfgrass is selected from the group consisting of *Agrostis spp.* (bentgrass species including colonial bentgrass and creeping bentgrasses), *Poa pratensis* (kentucky bluegrass), *Lolium spp.* (ryegrass species including annual ryegrass and perennial ryegrass), *Festuca arundinacea* (tall fescue) *Festuca rubra commutata* (fine fescue), *Cynodon dactylon* (common bermudagrass); *Pennisetum clandestinum* (kikuyugrass), *Stenotaphrum secundatum* (st. augustinegrass), *Zoysia japonica* (zoysiagrass), and *Dichondra micrantha*.

[0066] In another preferred embodiment, the germinating plant seedling is from a dicotyledenous plant. In one embodiment, the dicotyledenous plant is selected from the group consisting of cotton, tobacco, *Arabidopsis*, tomato, potato, sugar beet, broccoli, cassava, sweet potato, pepper, poinsettia, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, geranium, and cactus.

[0067] In another embodiment, the expression of the desired polynucleotide in the stably transformed plant confers a trait to the plant selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased

phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity, and production of novel proteins or peptides.

[0068] In a preferred embodiment, the desired polynucleotide is selected from the group consisting of a gene or part thereof, the 5'-untranslated region of the gene, the 3'-untranslated region of the gene, the leader sequence associated with the gene, or the trailer sequence associated with the gene.

[0069] In a preferred embodiment, the gene is selected from the group of genes encoding a peptide or protein displaying antifungal or antimicrobial activity such as alfalfa AFP and D4E1, a nutritional peptide or protein, a transcription factor such as CBF3, a receptor that binds to pathogen-derived ligands such as the disease resistance protein R1, a hemoglobin such as VhB, an oxidase such as polyphenol oxidase, an enzyme of the lignin biosynthesis pathway, an enzyme of industrial value, or an antigen.. Preferably, the desired polynucleotide is operably linked to a promoter and a terminator.

[0070] In a preferred embodiment, the sequences of the promoter and the terminator naturally occur in the genome of plants and organisms that produce, or are used in, edible food sources.

[0071] In one embodiment, a first vector carries the first T-DNA or P-DNA and a second vector carries the second T-DNA or P-DNA.

[0072] In one other embodiment, the second vector comprises at least one of an omega-mutated virD2 polynucleotide, a codA polynucleotide, and a codA::upp fusion polynucleotide.

[0073] The present invention contemplates transgenic plants and their progeny, that are produced by any of the methods described herein.

[0074] In another aspect of the invention, a method (“method 4”) for producing a transgenic plant is provided, comprising: (A) infecting a germinating plant seedling with an *Agrobacterium* transformation vector that comprises (i) a T-DNA or a P-DNA that comprises (a) the desired polynucleotide, and (b) a gene operably linked to a terminator that is not naturally expressed in plants, wherein the gene confers fertilizer tolerance to plants in which it is expressed; and (ii) a cytokinin gene, wherein the desired polynucleotide and the selectable marker gene are flanked by the border sequences of the T-DNA or by the border-like sequences of the P-DNA; (B) transferring the transformed seedling to soil and allowing them to grow into plants; (C) exposing the plants to 0.05% to 20% hydrogen cyanamide.

[0075] In one embodiment, the fertilizer tolerance gene confers resistance to cyanamide. In another embodiment, the selectable marker gene that confers resistance to cyanamide is selected from the group consisting of Cah, Cah homologs.

[0076] In another aspect, a method (“method 5”) is provided for producing a transgenic plant, comprising (a) vortexing a solution comprising a germinating plant seedling and at least one *Agrobacterium* strain that harbors a vector carrying a desired polynucleotide; (b) transferring the *Agrobacterium*-transformed seedling to soil, and exposing the transformed seedling to conditions that promote growth; and (d) screening the plants to determine if the desired polynucleotide is integrated into the genome of at least one cell of the plant, wherein a plant comprising the desired polynucleotide in the genome is a transgenic plant.

[0077] In one preferred embodiment the germinating plant seedling is from a monocotyledenous plant. In another embodiment, the monocotyledenous plant is selected from the group consisting of turfgrass, wheat, maize, rice, oat, barley, orchid, iris, lily, onion, and sorghum. In another embodiment, the turfgrass is selected from the group consisting of *Agrostis* spp. (bentgrass species including colonial bentgrass and creeping

bentgrasses), *Poa pratensis* (kentucky bluegrass), *Lolium spp.* (ryegrass species including annual ryegrass and perennial ryegrass), *Festuca arundinacea* (tall fescue) *Festuca rubra commutata* (fine fescue), *Cynodon dactylon* (common bermudagrass); *Pennisetum clandestinum* (kikuyugrass), *Stenotaphrum secundatum* (st. augustinegrass), *Zoysia japonica* (zoysiagrass), and *Dichondra micrantha*.

[0078] In another preferred embodiment, the germinating plant seedling is from a dicotyledenous plant. In one embodiment, the dicotyledenous plant is selected from the group consisting of cotton, tobacco, *Arabidopsis*, tomato, potato, sugar beet, broccoli, cassava, sweet potato, pepper, poinsettia, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, geranium, and cactus.

[0079] In another embodiment, the expression of the desired polynucleotide in the stably transformed plant confers a trait to the plant selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity, and production of novel proteins or peptides.

[0080] In a preferred embodiment, the desired polynucleotide of the present invention is selected from the group consisting of a gene or part thereof, the 5'-untranslated region of the gene, the 3'-untranslated region of the gene, the leader sequence associated with the gene, or the trailer sequence associated with the gene.

[0081] In a preferred embodiment, the gene is selected from the group of genes encoding a peptide or protein displaying antifungal or antimicrobial activity such as alfalfa AFP and D4E1, a nutritional peptide or protein, a transcription factor such as CBF3, a receptor that binds to pathogen-derived ligands such as the disease resistance protein R1, a hemoglobin such as VhB, an oxidase such as polyphenol oxidase, an enzyme of the lignin biosynthesis pathway, an enzyme of industrial value, or an antigen..

[0082] In a preferred embodiment, the sequences of the promoter and the terminator naturally occur in the genome of plants, or are isolated from human food sources.

[0083] In a preferred embodiment, the vector used in method 5 may be the one that is described in detail above.

[0084] In one other embodiment, the step of screening comprises detecting the presence of the desired polynucleotide in cells of the transgenic plant.

[0085] In another embodiment, the method further comprises producing progeny from the transgenic plant and detecting the presence of the desired polynucleotide in cells of the progeny. In another embodiment, the border-like sequences of the P-DNA range in size from 20 to 100 bp and share between 52% and 96% sequence identity with a T-DNA border sequence from *Agrobacterium tumefaciens*.

[0086] In another embodiment, expression of the selectable marker gene confers fertilizer tolerance to the transgenic plant and progeny thereof.

[0087] In another embodiment, the selectable marker gene that confers fertilizer tolerance is a selectable marker gene that confers resistance to cyanamide.

[0088] In another embodiment, the selectable marker gene that confers resistance to cyanamide is selected from the group consisting of Cah, Cah homologs. In another embodiment, the selectable marker gene is operably linked to a yeast ADH terminator. In another embodiment, the selectable marker gene is an antibiotic resistance gene. In yet another embodiment, the antibiotic resistance gene is selected from the group consisting of nptII or aph(3')II. In another embodiment, the selectable marker gene is a herbicide resistance gene. In another embodiment, the herbicide resistance gene is selected from the group consisting of GAT and EPSP synthase genes.

[0089] In one embodiment, the solution is vortexed from about 60 seconds to several hours. In another embodiment, the solution is vortexed for about 5 minutes to about 30 minutes.

[0090] In another aspect, a method ("method 6") is provided for producing a transgenic plant, comprising (a) vortexing a solution comprising a germinating plant seedling and at least one *Agrobacterium* strain that harbors a vector carrying a desired polynucleotide; (b) (i) producing callus from the transformed seedling; (ii) inducing shoot and root formation from the callus to produce a plantlet; (c) growing the plantlets into plants; and (d) screening the plants to determine if the desired polynucleotide is incorporated into the genome of at least one cell of the plant, wherein a plant comprising the desired polynucleotide in the genome is a transgenic plant.

[0091] In one preferred embodiment the germinating plant seedling is from a monocotyledenous plant. In another embodiment, the monocotyledenous plant is selected from the group consisting of turfgrass, wheat, maize, rice, oat, barley, orchid, iris, lily, onion, and sorghum. In another embodiment, the turfgrass is selected from the group consisting of *Agrostis* spp. (bentgrass species including colonial bentgrass and creeping bentgrasses), *Poa pratensis* (kentucky bluegrass), *Lolium* spp. (ryegrass species including annual ryegrass and perennial ryegrass), *Festuca*

arundinacea (tall fescue) *Festuca rubra commutata* (fine fescue), *Cynodon dactylon* (common bermudagrass); *Pennisetum clandestinum* (kikuyugrass), *Stenotaphrum secundatum* (st. augustinegrass), *Zoysia japonica* (zoysiagrass), and *Dichondra micrantha*.

[0092] In another preferred embodiment, the germinating plant seedling is from a dicotyledenous plant. In one embodiment, the dicotyledenous plant is selected from the group consisting of cotton, tobacco, *Arabidopsis*, tomato, potato, sugar beet, broccoli, cassava, sweet potato, pepper, poinsettia, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, geranium, and cactus.

[0093] In another embodiment, the expression of the desired polynucleotide in the stably transformed plant confers a trait to the plant selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity, and production of novel proteins or peptides.

[0094] In a preferred embodiment, the desired polynucleotide of the present invention is selected from the group consisting of a gene or part thereof, the 5'-untranslated region of the gene, the 3'-untranslated region of the gene, the leader sequence associated with the gene, or the trailer sequence associated with the gene.

[0095] In a preferred embodiment, the gene is selected from the group consisting of D4E1 synthetic peptide gene, HOS1 gene homologs, the *Vitreoscilla* hemoglobin gene, and genes involved in the lignin biosynthetic

pathway. Preferably, the desired polynucleotide is operably linked to a promoter and a terminator.

[0096] In a preferred embodiment, the sequences of the promoter and the terminator are isolated from the genome of human food sources.

[0097] In another embodiment, the vector comprises (a) a T-DNA or a P-DNA that comprises (i) the desired polynucleotide, and (ii) a selectable marker gene operably linked to a terminator that is not naturally expressed in plants; and (b) a backbone integration marker gene, wherein the desired polynucleotide and the selectable marker gene are positioned between the border sequences of the T-DNA or between the border-like sequences of the P-DNA, and wherein the backbone integration marker is not positioned within the T-DNA or within the P-DNA.

[0098] In another embodiment, the backbone integration marker gene is operably linked to a promoter and a terminator. In one embodiment, the backbone integration marker is a cytokinin gene. In yet another embodiment, the cytokinin gene is *IPT*, and the plant is a dicotyledon plant. In another embodiment, the backbone integration marker is *PGA22*, *TZS*, *HOC1*, *CKI1*, and *ESR1*.

[0099] In yet another embodiment, the border-like sequences of the P-DNA range in size from 20 to 100 bp and share between 52% and 96% sequence identity with a T-DNA border sequence from *Agrobacterium tumefaciens*.

[0100] In another embodiment, expression of the selectable marker gene confers fertilizer tolerance to the transgenic plant and progeny thereof.

[0101] In another embodiment, the selectable marker gene that confers fertilizer tolerance is a selectable marker gene that confers resistance to cyanamide.

[0102] In another embodiment, the selectable marker gene that confers resistance to cyanamide is selected from the group consisting of *CAH* or *CAH* homologs derived from certain cyanamide tolerant soil fungi including *Aspergillus*, *Penicillium*, and *Cladosporium*. In another embodiment, the selectable marker gene is operably linked to a yeast ADH terminator. In another embodiment, the selectable marker gene is an antibiotic resistance gene. In yet another embodiment, the antibiotic resistance gene is selected from the group of genes encoding hygromycin phosphotransferase, neomycin phosphotransferase, streptomycin phosphotransferase, and bleomycin-binding protein.. In another embodiment, the selectable marker gene is a herbicide resistance gene. In another embodiment, the herbicide resistance gene is selected from the group of genes encoding 5-enolpyruvylshikimate-3-phosphate synthase, glyphosate oxidoreductase, glyphosate-N-acetyltransferase, and phosphinothricin acetyl transferase.

[0103] In another embodiment, the step of screening comprises detecting the presence of the desired polynucleotide in cells of the transgenic plant.

[0104] In another embodiment, the method comprises producing progeny from the transgenic plant and detecting the presence of the desired polynucleotide in cells of the progeny.

[0105] In one other embodiment, the solution is vortexed from about 60 seconds to several hours. In another embodiment, the solution is vortexed for about 5 minutes to about 30 minutes.

[0106] The method, in another embodiment, further comprises the step of growing the seedling of (e) into a plant, wherein the plant is a transformed plant and wherein at least one cell of the transformed plant comprises in its genome the desired polynucleotide.

[0107] In another embodiment, the method further comprises crossing the transformed plant with a non-transformed plant to produce at

least one progeny plant that comprises the desired polynucleotide in its genome.

[0108] In another embodiment, the method further comprises selfing the transformed plant to produce at least one progeny plant that comprises the desired polynucleotide in its genome.

[0109] According to the invention, the desired polynucleotide is operably linked to a promoter and a terminator. In one embodiment, the desired polynucleotide consists essentially of a sequence that is native to the selected plant, native to a plant from the same species, or is native to a plant that is sexually interfertile with the selected plant. In another embodiment, the desired polynucleotide, the promoter, and the terminator consist essentially of sequences that are endogenous to a sequence naturally found in a plant or derived from a food source.

[0110] In another embodiment, the modification of expression of a functional gene results in the modification of a trait to plants that comprise the desired polynucleotide in their genomes, wherein the trait is selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity, and production of novel proteins or peptides.

[0111] In one other embodiment, the first vector and the second vector are both present in the same strain of *Agrobacterium*.

[0112] In another embodiment, the first vector is present in a first strain of *Agrobacterium* and the second vector is present in a second, different strain of *Agrobacterium*

[0113] In another aspect, the invention provides a method (“method 7”) for identifying promoters that function in plant cells, comprising:

[0114] (a) creating *Agrobacterium* binary vectors that each comprise an plant-derived polynucleotide that is operably linked to a Cah gene;

[0115] (b) infecting a germinating plant seedling with *Agrobacterium* strains comprising the binary vectors;

[0116] (c) transferring the transformed seedling to media that comprises cyanamide and allowing the seedling to form calli, wherein only seedling that can express the Cah gene will form calli;

[0117] (d) transferring cyanamide resistant calli to shoot-inducing medium, and isolating DNA from resultant shoots; and

[0118] (e) identifying the sequence of the artificial polynucleotide driving expression of the Cah gene, wherein the sequence of the plant-derived polynucleotide represents the sequence of a synthetic promoter.

[0119] In another embodiment, the present invention contemplates a CAH gene homolog with the sequence of SEQ ID NO. 1, and variants thereof, which confer resistance to cyanamide.

[0120] In another embodiment, the present invention encompasses a terminator sequence that is associated with the rice actin-1 gene described in SEQ ID NO. 6, and variants thereof, which function as a terminator.

[0121] In another embodiment, the present invention contemplates a plant-like promoter gene with the sequence of SEQ ID NO. 9, and variants thereof, which function as a promoter.

[0122] Thus, the present invention encompasses a polynucleotide that has a sequence identity that is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% in sequence to SEQ ID NO. 1, and which encodes a protein that is cyanamide tolerant. Variants that have less than 60% sequence identity to SEQ ID NO. 1, but which also encode functional cyanamide tolerant proteins are also encompassed by the present invention.

[0123] The present invention encompasses a polynucleotide that has a sequence identity that is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% in sequence to SEQ ID NO. 6, and which encodes a functional terminator. Variants that have less than 60% sequence identity to SEQ ID NO. 6, but which also encode functional terminators are also encompassed by the present invention.

[0124] The present invention encompasses a polynucleotide that has a sequence identity that is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% in sequence to SEQ ID NO. 9, and which encodes a promoter that is functional in plants. Variants that have less than 60% sequence identity to SEQ ID NO. 9, but which also encode functional promoters are also encompassed by the present invention.

[0125] The present invention encompasses a polynucleotide that has a sequence identity that is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%,

69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% in sequence to any one of SEQ ID NOs. 12, 14, or 15, *i.e.*, a cyanamide resistance gene, and which encodes a functional cyanamide resistance protein. Variants that have less than 60% sequence identity to any one of SEQ ID NOs. 12, 14, or 15, but which also encode functional cyanamide resistance proteins, are also encompassed by the present invention. A particular nucleic acid sequence, such as any of those described herein, also implicitly encompasses conservatively modified variants thereof and complementary sequences, as well as the sequence explicitly indicated. That is, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19: 5081 (1991); Ohtsuka et al., J. Biol. Chem. 260: 2605-2608 (1985); Rossolini et al., Mol. Cell. Probes 8: 91-98 (1994)). The terms "nucleic acid" or "nucleic acid sequence" or "polynucleotide" may also be used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0126] Preferably, the polynucleotide of SEQ ID NOs. 12, 14, or 15, or a variant thereof, encodes a cyanamide resistance protein, especially one that comprises the amino acid sequence depicted in SEQ ID NO. 13. Thus, the present invention encompasses amino acid variants of the sequence of SEQ ID NO. 13, so long as the resultant cyanamide resistance protein is still capable of conferring cyanamide resistance. Accordingly, the present invention encompasses a polypeptide that has a sequence identity that is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% in sequence to the amino acid sequence of SEQ ID NO. 13.

[0127] A polynucleotide sequence that encodes such a protein, *i.e.*, any one of those depicted in SEQ ID NOs. 12, 14, and 15, can be "codon-optimized" so that it is more suitably transcribed and translated, *i.e.*, better

expressed, in a particular plant type or species. That is, codon optimization favors maximum protein expression by increasing the translational efficiency of a particular gene. "Protein engineering" and "protein evolution" are terms synonymous with such codon modification processes. For instance, the present invention contemplates optimizing one or more codons of SEQ ID NO. 12 so that the ultimate nucleotide sequence is optimized for expression in either a monocotyledonous or dicotyledonous plant. For example, the polynucleotide sequence depicted in SEQ ID NO. 14 has been codon optimized from the base sequence of SEQ ID NO. 12, so that the polynucleotide of SEQ ID NO. 12 is more efficiently expressed in a monocotyledonous plant. Similarly, SEQ ID NO. 12 has been codon optimized to produce the sequence depicted in SEQ ID NO. 15, which is optimized for expression in a dicotyledonous plant.

[0128] The present invention also encompasses a polynucleotide comprises the sequence of any one of SEQ ID NOs. 1, 6, or 9. Furthermore, the present invention encompasses a polynucleotide consisting essentially of the sequence of any one of SEQ ID NOs. 1, 6, or 9. Finally, the present invention encompasses a polynucleotide consisting of the sequence of any one of SEQ ID NOs. 1, 6, or 9.

[0129] Thus, the present invention encompasses the use of the rice actin-1 terminator sequence (SEQ ID NO. 6) in a construct, operably linked to a desired polynucleotide, to terminate expression of a desired polynucleotide. Similarly, the sugarcane-like promoter (SEQ ID NO. 9) can be operably linked to a desired polynucleotide to express the desired polynucleotide.

[0130] In one other embodiment, the efficiency of stable transformation can be further enhanced by inducing double strand breaks in the chromosomes of germinating seedling before, during, and/or after infection. For instance, a plant tissue may be exposed to such a chemical compound one day prior to infection, and then again after infection for about 1 hour, about 2 or more hours, about 5 or more hours, about 10 or more hours,

or one or more days. In one embodiment, double strand breaks are generated by subjecting seedlings to low doses of chemicals such as methyl methane sulfonate (MMS), HO-endonuclease, bleomycin, neocarzinostatin, camptothecan, and cisplatin. In another embodiment, the seedling is exposed, before, during, or after infection to ionizing radiation or heavy ions.

[0131] Accordingly, in another aspect, methods of the present invention can be adapted to include a step that induces a double strand break in the plant genome in order to increase the frequency of integration of the desired polynucleotide. In one embodiment, the inventive methodology may entail vortexing a plant tissue with an *Agrobacterium* vector to optimize transfer of the vector and desired polynucleotide(s) into plant cells, and also the induction of double stranded breaks in plant chromosomes to increase the frequency of stably transforming, *i.e.*, integrating, the plant genome with the desired polynucleotide(s).

[0132] In another embodiment, the present invention is not limited to the transfer of nucleic acids into a plant cell by *Agrobacterium*-mediated transformation methods. Other methods, such as the inventive vortexing method, particle bombardment, polyethylene glycol treatment, liposomal delivery, microinjection, whiskers, and electroporation can be used in conjunction with the chemical compounds, or ionizing radiation or heavy ion exposure, described above for inducing double strand breaks in the plant chromosomal DNA. Accordingly, the present invention is not limited to only the combination of vortexing and induction of double strand breaks. For example, plant tissues may be transformed using whiskers combined with exposure to methyl methane sulfonate.

[0133] Furthermore, the DNA and/or desired polynucleotide to be transferred into the plant cell can be in the form of naked DNA, plasmid DNA, liposomal DNA, or coated onto beads, particles, whiskers, needles, or in any other formulation known to the skilled artisan.

[0134] The present invention provides another method for producing a transgenic plant, comprising (a) agitating a solution, which comprises (1) a germinating plant seedling, or explant thereof, and (2) at least one *Agrobacterium* strain that comprises a vector, which comprises a desired polynucleotide; (b) cultivating the seedling to produce a plant; and (c) screening the plant to determine if the desired polynucleotide is integrated into the genome of at least one cell of the plant to produce a stably transformed plant, wherein the step of agitating the solution does not comprise sonication, and wherein the germinating plant seedling is exposed to an agent that enhances transformation efficiency before, during, or after the step of agitating the solution.

[0135] According to this method, the agent that enhances transformation efficiency is at least one of a purine inhibitor, a pyrimidine inhibitor, or a purine- and a pyrimidine- inhibitor. In a preferred embodiment, the agent is selected from the group consisting of mizoribine, azathioprine, mycophenolic acid, mycophenolate mofetil, 5-fluorouracil, Brequinar sodium, leflunomide, azaserine, acivicin, methotrexate, methotrexate polyglutamate derivatives, and cyclophosphamide. In another embodiment, the agent is a purine inhibitor and a pyrimidine inhibitor. Preferably, the agent is azaserine or acivicin.

[0136] In yet another embodiment, the agent induces chromosome breakage. Preferably, the agent is methyl methane sulfonate.

[0137] According to such a method, the vector comprises (a) a T-DNA or a P-DNA, which comprises (i) the desired polynucleotide, and (ii) a selectable marker gene operably linked to a terminator that is not naturally expressed in plants; and (b) a backbone integration marker gene, wherein the desired polynucleotide and the selectable marker gene are positioned between the border sequences of the T-DNA or between the border-like sequences of the P-DNA, and wherein the backbone integration marker gene is not positioned within the T-DNA or within the P-DNA.

[0138] In one embodiment, the method further comprises

(i) producing a callus from the cultivated seedling; and (ii) inducing shoot and root formation from the callus, prior to transferring to soil to produce the plant. In another embodiment, the step of producing the callus from the transformed seedling comprises (i) transferring the seedling that had been subjected to agitation to tissue culture media, which contains auxin and cyanamide; (ii) selecting a fertilizer-resistant callus; (iii) inducing shoot and root formation from the callus; and (iv) transferring a callus with shoots and roots to soil and exposing the callus to conditions that promote growth of a transgenic plant from the callus. Preferably, expression of the selectable marker gene confers fertilizer resistance or cyanamide resistance to the transgenic plant and to progeny of the transgenic plant

[0139] In a preferred embodiment, the selectable marker gene is a cyanamide resistance gene. In a further preferred embodiment, the cyanamide resistance gene comprises the nucleotide sequence depicted in any one of SEQ ID NO. 12 or a variant thereof, SEQ ID NO. 14 or a variant thereof, or SEQ ID NO. 15 or a variant thereof, and wherein the gene encodes a protein that confers cyanamide resistance. In another embodiment, the protein that confers cyanamide resistance comprises the sequence of SEQ ID NO. 13 or a variant thereof, wherein the variant protein is functionally active.

[0140] According to another embodiment of this method, the germinating plant seedling or explant thereof is a monocotyledonous plant and the cyanamide resistance gene (i) comprises the sequence of SEQ ID NO. 14, or a variant thereof, and (ii) encodes a functional cyanamide resistance protein.

[0141] In yet another embodiment, the plant seedling or explant thereof is a dicotyledonous plant and the cyanamide resistance gene (i) comprises the sequence of SEQ ID NO. 15, or a variant thereof, and (ii) encodes a functional cyanamide resistance protein.

[0142] In a further embodiment, the germinating plant seedling is from a monocotyledonous plant. In a preferred embodiment, the monocotyledonous plant is selected from the group consisting of bentgrass, bluegrass, turfgrass, wheat, maize, rice, oat, barley, orchid, iris, lily, onion, sugarcane, and sorghum. In another embodiment, the turfgrass is selected from the group consisting of *Agrostis spp.*, *Poa pratensis*, *Lolium spp.*, *Festuca arundinacea*, *Festuca rubra commutata*, *Cynodon dactylon*, *Pennisetum clandestinum*, *Stenotaphrum secundatum*, *Zoysia japonica*, and *Dichondra micrantha*.

[0143] In a further embodiment of this method, the germinating plant seedling is from a dicotyledonous plant. Preferably, the dicotyledonous plant is selected from the group consisting of cotton, tobacco, *Arabidopsis*, tomato, potato, sugar beet, broccoli, cassava, sweet potato, pepper, poinsettia, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, geranium, and cactus.

[0144] In another embodiment of this method, expression of the desired polynucleotide in the stably transformed plant confers a trait to the plant selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity, and production of novel proteins or peptides.

[0145] In a further embodiment, the desired polynucleotide expresses a peptide or protein that is an antifungal, a nutritional peptide or protein, a transcription factor, a receptor that binds to pathogen-derived

ligands, a hemoglobin, an oxidase, an enzyme of the lignin biosynthesis pathway, an enzyme of industrial value, or an antigen.

[0146] In yet another embodiment, the backbone integration marker is a cytokinin gene. Preferably, the cytokinin gene is *IPT*, and the plant is a dicotyledonous plant. In a more preferred embodiment, the backbone integration marker is *PGA22*, *TZS*, *HOC1*, *CKI1*, and *ESR1*.

[0147] According to this method, the step of agitating the solution is accomplished by vortexing. In one embodiment, the solution is vortexed from about 60 seconds to several hours. In a preferred embodiment, the solution is vortexed for about 5 minutes to about 30 minutes.

[0148] In yet another aspect of this method, the step of cultivating the seedling to produce a transgenic plant comprises transferring the *Agrobacterium*-transformed seedling to soil, and exposing the transformed seedling to conditions that promote growth. In one embodiment, the step of cultivating the seedling to produce a transgenic plant comprises cultivating the *Agrobacterium*-transformed seedling in or on tissue culture medium prior to transferring the transformed seedling to soil, and exposing the transformed seedling to conditions that promote growth.

[0149] In another embodiment, the transformed plant seedling is grown to maturity, crossed to a non-transformed plant and the desired polynucleotide transmitted to at least one progeny plant.

[0150] In yet another embodiment, the transformed plant seedling is grown to maturity, selfed, and the desired polynucleotide transmitted to progeny.

[0151] In another aspect of the present invention, a method for producing a transgenic plant is provided, which comprises (a) agitating a solution that comprises (1) a germinating plant seedling and (2) at least one *Agrobacterium* strain that comprises a vector, which comprises (i) a desired

polynucleotide and (ii) a cyanamide resistance gene; (b) (i) producing a callus from the transformed seedling and (ii) inducing shoot and root formation from the callus to produce plantlets; (c) growing the plantlets into plants; and (d) screening the plants to determine if the desired polynucleotide is incorporated into the genome of at least one cell of the plant to produce a stably transformed transgenic plant, and wherein the step of agitating the solution does not comprise sonication.

[0152] In a preferred embodiment, the desired polynucleotide and the cyanamide resistance gene, which is operably linked to a terminator that is not naturally expressed in plants, are positioned between border or border-like sequences of a T-DNA or a P-DNA located in the vector.

[0153] In a more preferred embodiment, the cyanamide resistance gene comprises the nucleotide sequence of any one of SEQ ID NOs. 12 or a variant thereof, SEQ ID NO. 14 or a variant thereof, or SEQ ID NO. 15 or a variant thereof, and wherein the cyanamide resistance gene encodes a protein that comprises the amino acid sequence of SEQ ID NO. 13.

[0154] According to this method, the vector further comprises a backbone integration marker gene, which is not positioned between the border or border-like sequences of the T-DNA or the P-DNA.

[0155] In yet another embodiment, this method further comprises exposing the germinating plant seedling to an agent that enhances transformation efficiency. In a preferred embodiment, the agent that enhances transformation efficiency is at least one of a purine inhibitor, a pyrimidine inhibitor, or a purine- and a pyrimidine- inhibitor. In a more preferred embodiment, the agent is selected from the group consisting of mizoribine, azathioprine, mycophenolic acid, mycophenolate mofetil, 5-fluorouracil, Brequinar sodium, Leflunomide, azaserine, acivicin, methotrexate, methotrexate polyglutamate derivatives, and cyclophosphamide.

[0156] In a further embodiment, the agent induces chromosomal breakage. Preferably, the agent is methyl methane sulfonate.

[0157] In another embodiment, the step of agitating the solution is accomplished by vortexing. Preferably, the solution is vortexed from about 60 seconds to several hours. In a more preferred embodiment, the solution is vortexed for about 5 minutes to about 30 minutes.

[0158] In another aspect of the present invention, an isolated nucleic acid is provided, which comprises the sequence of SEQ ID NO. 12, or variant thereof, wherein the nucleic acid encodes a functional cyanamide resistance protein. In a preferred embodiment, a variant of SEQ ID NO. 12 has a sequence identity of at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% to the nucleic acid sequence of SEQ ID NO. 12. In a preferred embodiment, the isolated nucleic acid of claim 45, wherein the cyanamide resistance protein comprises the amino acid sequence of SEQ ID NO. 13 or variant thereof.

[0159] In another embodiment, an isolated nucleic acid is provided, which comprises the sequence of SEQ ID NO. 14, or variant thereof, wherein the nucleic acid encodes a functional cyanamide resistance protein. In a preferred embodiment, a variant of SEQ ID NO. 14 has a sequence identity of at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at

least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% to the nucleic acid sequence of SEQ ID NO. 14. In a preferred embodiment, the cyanamide resistance protein comprises the amino acid sequence of SEQ ID NO. 13 or variant thereof.

[0160] Also provided is an isolated nucleic acid, which comprises the sequence of SEQ ID NO. 15, or variant thereof, wherein the nucleic acid encodes a functional cyanamide resistance protein. In a preferred embodiment, a variant of SEQ ID NO. 15 has a sequence identity of at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 770%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% to the nucleic acid sequence of SEQ ID NO. 15. In a preferred embodiment, the cyanamide resistance protein comprises the amino acid sequence of SEQ ID NO. 13 or variant thereof.

[0161] In yet another aspect, an isolated cyanamide resistance protein is provided, comprising the amino acid sequence of SEQ ID NO. 13, or variants thereof, wherein the protein confers resistance to cyanamide. In a preferred embodiment, a variant of SEQ ID NO. 13 has a sequence identity of at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 770%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at

least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% to the amino acid sequence of SEQ ID NO. 13.

BRIEF DESCRIPTION OF THE DRAWINGS

[0162] Figure 1: Schematic flowchart of the inventive methods and compositions.

[0163] Figure 2: Alignment of the *CAH* gene from *Myrothecium verrucaria* with a new cyanamide tolerance gene isolated from *Aspergillus* (CAH-H1) and a non-functional yeast *CAH* homolog (CAH-H2).

[0164] Figure 3: Alignment between a new ubiquitin-like promoter (UbiN) and the corresponding partial sequence of the sugarcane Ubiquitin-4 promoter.

[0165] Figure 4: GUS-expression in *Agrobacterium*-infected alfalfa seedlings: Expression is indicated as percentage of plant surface displaying GUS activity, 5 days after subjection to *Agrobacterium*.

“- vortex”: subjection by immersing (30 minutes) seedlings into an *Agrobacterium* suspension; “+ vortex” subjection by vortexing (30 minutes) seedlings immersed in an *Agrobacterium* suspension. The over-all difference in transformation efficiency between immersion and vortex-mediated transformation is greater than 50-fold.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0166] The present invention provides methods for producing transgenic plants and transformation vectors.

[0167] The present inventive methods can be applied to many species of plants, including those that are difficult to transform by applying conventional transformation methods. The present invention provides methods for integrating a desired polynucleotide into a plant genome to alter the expression of a plant trait, or to produce a product, such as a pharmaceutically relevant or important protein, and methods for readily selecting and screening for cells and plants that comprise the desired polynucleotide in their genome.

[0168] In particular, the inventive transformation methods include transforming germinating seedling with a vector comprising a desired polynucleotide, and then either (1) planting the seedling directly into soil; (2) transferring the seedling to culture media, without inducing a callus phase, and then planting the seedling directly into soil; or (3) transferring the seedling to culture media, inducing a callus phase, and shoot and root formation, and then planting the seedling directly into soil.

[0169] Figure 1 illustrates such methods. Plant tissues (Figure 1, box "(a)") may be transformed by vortexing (Figure 1, box "(c)"), and then planted directly into soil (Figure 1, box "(e)") and then grown into the desired transgenic plant (Figure 1, box "(h)").

[0170] Alternatively, after vortexing (Figure 1, box "(c)'), the transformed plant tissues may be nurtured on tissue culture medium (Figure 1, box "(d)'), planted directly into soil (Figure 1, box "(e)'), and then grown into the desired transgenic plant (Figure 1, box "(h)').

[0171] Finally, after vortexing, and nurturing on tissue culture medium, the plant tissue can be induced to undergo callus formation (Figure

1, box "(g)", and shoot and root growth, prior to being grown into the desired transgenic plant.

[0172] The inventive *Agrobacterium* vector that can be used in any one of such methods is illustrated in Figure 1, box "(f)". The vector may, or may not, include a selectable/screenable marker for identifying transformed, transgenic plants, parts thereof, or transformed cells. For instance, when a plant tissue, such as a seedling, is transformed according to the present methods and planted directly into soil without the induction of a callus phase, the vector does not need to contain a selectable marker gene. Alternatively, after culturing the transformed seedling, or other plant tissue, on callus-inducing tissue culture media, one may select for successful transformants by including in the tissue media, a substance(s) to which only plant cells that contain a selectable marker gene are resistant to, or can tolerate.

[0173] The present invention uses terms and phrases that are well known to those practicing the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described herein are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, microbial culture, cell culture, tissue culture, transformation, transfection, transduction, analytical chemistry, organic synthetic chemistry, chemical syntheses, chemical analysis, and pharmaceutical formulation and delivery. Generally, enzymatic reactions and purification and/or isolation steps are performed according to the manufacturers' specifications. The techniques and procedures are generally performed according to conventional methodology (Sambrook & Russel, MOLECULAR CLONING: A LABORATORY MANUAL, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001).

[0174] *Agitation:* “agitation” means to cause movement with violence or sudden force. With respect to the present invention, “agitation” refers to a violent and sudden physical vibration of a solution. “Agitation,” as used herein, does not encompass the disruption of a solution by treatment with high-frequency sound waves, such as those produced by sonication.

[0175] *Agrobacterium:* as is well known in the field, *Agrobacteria* that are used for transforming plant cells, are disarmed and virulent derivatives of, usually, *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* that contain a vector. The vector typically contains a desired polynucleotide that is located between the borders of a T-DNA or, according to the present invention, between the border-like sequences of a “plant-DNA” (“P-DNA”), see definition below, which border (like) sequences are capable of transferring the desired polynucleotide into a plant genome.

[0176] *Border and Border-like sequences:* “border sequences” are specific *Agrobacterium*-derived sequences. Typically, a left border sequence and a right border sequence flank a T-DNA and function as recognition sites for *virD2*-catalyzed nicking reactions. The sequences of the left and right border sequences may or may not be identical. Their sequences may or may not be inverted repeats of one another. Such activity releases nucleic acid that is positioned between such borders. See Table 1 below for examples of border sequences. The released nucleic acid, complexed with *virD2* and *virE2*, is targeted to plant cell nuclei where the nucleic acid is often integrated into the genome of the plant cell. Usually, two border sequences, a left-border and a right-border, are used to integrate a nucleotide sequence that is located between them into another nucleotide sequence. It is also possible to use only one border, or more than two borders, to accomplish integration of a desired nucleic acid in such fashion.

[0177] According to the present invention, a “border-like” sequence is isolated from a plant, and functions like the border sequence of an *Agrobacterium*-derived T-DNA. That is, a border-like sequence of the present

invention promotes and facilitates the transfer of a polynucleotide to which it is linked from *Agrobacterium* to plant cell nuclei, and the subsequent stable integration of this polynucleotide into the plant genome. A plant-DNA, i.e., P-DNA, of the present invention preferably is delineated by border-like sequences.

[0178] A border-like sequence of a P-DNA is between 5-100 bp in length, 10-80 bp in length, 15-75 bp in length, 15-60 bp in length, 15-50 bp in length, 15-40 bp in length, 15-30 bp in length, 16-30 bp in length, 20-30 bp in length, 21-30 bp in length, 22-30 bp in length, 23-30 bp in length, 24-30 bp in length, 25-30 bp in length, or 26-30 bp in length.

[0179] The border-like sequences of the present invention can be isolated from any plant. See SEQ ID NO.: 3 for a DNA fragment isolated from potato that contains, at either end, a border-like sequence. Thus, P-DNA border-like sequences of use for the present invention are isolated from a plant. A P-DNA border-like sequence is not identical in nucleotide sequence to any known *Agrobacterium*-derived T-DNA border sequence. Thus, a P-DNA border-like sequence may possess 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more nucleotides that are different from a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. That is, a P-DNA border, or a border-like sequence of the present invention has at least 95%, at least 90%, at least 80%, at least 75%, at least 70%, at least 60% or at least 50% sequence identity with a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, but not 100% sequence identity. As used herein, the descriptive terms "P-DNA border" and "P-DNA border-like" are exchangeable.

[0180] A native P-DNA border sequence is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%,

60%, 59%, 58%, 57%, 56%, 55%, 54%, 53%, 52%, 51% or 50% similar in nucleotide sequence to a *Agrobacterium* a T-DNA border sequence. A border-like sequence can, therefore, be isolated from a plant genome and be modified or mutated to change the efficiency by which they are capable of integrating a nucleotide sequence into another nucleotide sequence. Other polynucleotide sequences may be added to or incorporated within a border-like sequence of the present invention. Thus, a P-DNA left border or a P-DNA right border may be modified so as to possess 5'- and 3'- multiple cloning sites, or additional restriction sites. A P-DNA border sequence may be modified to increase the likelihood that backbone DNA from the accompanying vector is not integrated into the plant genome.

[0181] Table 1 below depicts the sequences of known T-DNA border sequences and sequences identified herein as border-like sequences. By aligning sequences with known T-DNA border sequences, new "border-like" sequences were identified that existed in plant genomes. The "potato" border-like sequences of Table 1 were isolated herein, using degenerate primers in polymerase chain reactions on potato genomic template DNA. The present invention encompasses the use of such potato P-DNA border-like elements for transferring a desired polynucleotide into the genome of a plant cell.

Table 1. "Border" and "Border-Like" sequences

<i>Agrobacterium</i> T-DNA borders	
TGACAGGATATAATTGGCGGGTAAAC (SEQ ID NO.12)	<i>Agro.</i> nopaline strains (RB)
TGGCAGGATATAATTGTGGTGTAAAC (SEQ ID NO.13)	<i>Agro.</i> nopaline strains (LB)
TGGCAGGATATAACCGTTGTAAATT (SEQ ID NO.14)	<i>Agro.</i> octopine strains (RB)
CGGCAGGATATAATTCAATTGTAAATT (SEQ ID NO.15)	<i>Agro.</i> octopine strains (LB)
TGGTAGGATATAACCGTTGTAAATT (SEQ ID NO.16)	LB mutant
TGGCAGGATATACTGGTACTGTAAATT (SEQ ID NO.17)	LB mutant
YGRYAGGATATAWSNVBKGTAAWY (SEQ ID NO.18)	Border motif
Border-like sequences	
TGACAGGATATACTGTAAAC (SEQ ID NO.19)	potato (border-like sequence)*
TGGCAGGATATAACCGATGTAAAC (SEQ ID NO.20)	potato (border-like sequence)*

Y= C or T; R= A or G; K= G or T; M= A or C; W= A or T; S= C or G; V= A, C, or G; B= C, G, or T.

*potato border-like sequences were obtained and isolated according to the presently-described inventive methods.

[0182] ***Callus formation:*** typically, young roots, stems, buds, and germinating seedlings are a few of the sources of plant tissue that can be used to induce callus formation. Callus formation is controlled by growth regulating substances present in tissue culture medium, such as auxins and cytokinins. The specific substances, and concentrations of those substances, that induce callus formation varies between plant species. Occassionally, different sources of explants require different culturing conditions, even if obtained from the same plant or species. Accordingly, a cocktail of various growth substances can be added to tissue culture medium in order to induce callus formation from a variety of plant species that are incubated on such media. Other factors, such as the amount of light, temperature, and humidity, for instance, are important in establishing a callus. Once established, callus cultures can be used to obtain protoplasts, or study somatic embryogenesis, organogenesis, and secondary metabolite production.

[0183] The skilled artisan is well aware of various protocols, media, and conditions that can be modified to induce callus formation from a particular explant. The FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS' Agricultural Services Bulletin No. 108, entitled, "PLANT TISSUE CULTURE: AN ALTERNATIVE FOR PRODUCTION OF USEFUL METABOLITE" by Masanaru Misawa of Bio International Inc., Toronto, Canada (<http://www.fao.org/docrep/t0831e/t0831e00.htm#con>) lists such conditions in Chapter 4. There, one learns that the successful production of callus depends upon plant species and their qualities. Dicotyledons, for example, are quite amenable to callus formation, compared to monocotyledons. Suitable tissue culture media for inducing callus formation from an explant may include inorganic salts, carbon sources, vitamins, phytohormones, and organic supplements. See for additional information: Plant Cell Tissue and Organ Culture, Fundamental Methods, Gamborg and Phillips, eds, 1995 (Springer Verlag, New York)

[0184] Desired Polynucleotide: a desired polynucleotide of the present invention is a genetic element, such as a promoter, enhancer, or terminator, or gene or polynucleotide that is to be transcribed and/or translated in a transformed cell that comprises the desired polynucleotide in its genome. If the desired polynucleotide comprises a sequence encoding a protein product, the coding region may be operably linked to regulatory elements, such as to a promoter and a terminator, that bring about expression of an associated messenger RNA transcript and/or a protein product encoded by the desired polynucleotide. Thus, a "desired polynucleotide" may comprise a gene that is operably linked in the 5'- to 3'- orientation, a promoter, a gene that encodes a protein, and a terminator. Alternatively, the desired polynucleotide may comprise a gene or fragment thereof, in an "antisense" orientation, the transcription of which produces nucleic acids that may form secondary structures that affect expression of an endogenous gene in the plant cell. A desired polynucleotide may also yield a double-stranded RNA product upon transcription that initiates RNA interference of a gene to which the desired polynucleotide is associated. A desired polynucleotide of the present invention may be positioned within a T-DNA or P-DNA, such that the left and right T-DNA border sequences, or the left and right border-like sequences of the P-DNA, flank or are on either side of the desired polynucleotide. The present invention envisions the stable integration of one or more desired polynucleotides into the genome of at least one plant cell. A desired polynucleotide may be mutated or a variant of its wild-type sequence. It is understood that all or part of the desired polynucleotide can be integrated into the genome of a plant. It also is understood that the term "desired polynucleotide" encompasses one or more of such polynucleotides. Thus, a P-DNA or T-DNA of the present invention may comprise one, two, three, four, five, six, seven, eight, nine, ten, or more desired polynucleotides.

[0185] According to the present invention, a desired polynucleotide also may be used to alter a trait (see definition below) associated with a plant. In a situation where the plant is a food crop for consumption, it is preferable that

the plant is not transformed so as to integrate undesirable DNA into its genome. A desired polynucleotide also may be used for pharmaceutical purposes, to express in plants a product of pharmaceutical relevance or importance. In that situation, any foreign, native, or undesirable nucleic acids may be used to express the desired polynucleotide. Examples of pharmaceutically relevant desired polynucleotides include those that encode peptides, nutraceuticals, vaccines, growth factors, and enzymes.

[0186] *Dicotyledonous plant (dicot)*: a flowering plant whose embryos have two seed halves or cotyledons. Examples of dicots include but are not limited to, cotton, tobacco, *Arabidopsis*, tomato, potato sugar beet, broccoli, cassava, sweet potato, pepper, poinsettia, bean, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, geranium, avocado, and cactus.

[0187] *Food source*: the present invention contemplates to improve food crops by introducing DNA that is mainly or exclusively derived from human food sources into the genomes of these crops and plants. Examples of edible food sources preferably includes baker's yeast and plants that produce edible fruits, vegetables, and grains. Preferably, DNA is not obtained from animals, bacteria, viruses, and fungi. Accordingly, genetic elements such as promoters, terminators, genes, and selectable markers, introduced into a plant genome, may be preferably derived from, or isolated from, plants that produce edible foods or organisms, such as yeast.

[0188] *Foreign*: "foreign," with respect to a nucleic acid, means that that nucleic acid is derived from non-plant organisms, or derived from a plant that is not the same species as the plant to be transformed or is not derived from a plant that is not interfertile with the plant to be transformed, does not belong to the species of the target plant. According to the present invention, foreign DNA or RNA represents nucleic acids that are naturally occurring in the genetic makeup of fungi, bacteria, viruses, mammals, fish or birds, but are not naturally occurring in the plant that is to be transformed. Thus, a foreign

nucleic acid is one that encodes, for instance, a polypeptide that is not naturally produced by the transformed plant. A foreign nucleic acid does not have to encode a protein product. According to the present invention, a most desired transgenic plant is one that contains minimal, if any, foreign nucleic acids integrated into its genome. The present invention also encompasses transgenic plants that do contain non-plant species nucleic acids in their genomes.

[0189] Gene: A gene is a segment of a DNA molecule that contains all the information required for synthesis of a product, polypeptide chain or RNA molecule, that includes both coding and non-coding sequences.

[0190] Genetic element: a "genetic element" is any discreet nucleotide sequence such as, but not limited to, a promoter, gene, terminator, intron, enhancer, spacer, 5'-untranslated region, 3'-untranslated region, or recombinase recognition site.

[0191] Genetic modification: stable introduction of DNA into the genome of certain organisms by applying methods in molecular and cell biology.

[0052] Introduction: as used herein, refers to the insertion of a nucleic acid sequence into a cell, by methods including infection, transfection, transformation or transduction.

[0192] Monocotyledonous plant (monocot): a flowering plant whose embryos have one cotyledon or seed leaf. Examples of monocots include, but are not limited to turfgrass, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, and palm. Examples of turfgrass include, but are not limited to *Agrostis spp.* (bentgrass species including colonial bentgrass and creeping bentgrasses), *Poa pratensis* (kentucky bluegrass), *Lolium spp.* (ryegrass species including annual ryegrass and perennial ryegrass), *Festuca arundinacea* (tall fescue) *Festuca rubra commutata* (fine

fescue), *Cynodon dactylon* (common bermudagrass varieties including Tifgreen, Tifway II, and Santa Ana, as well as hybrids thereof); *Pennisetum clandestinum* (kikuyugrass), *Stenotaphrum secundatum* (st. augustinegrass), *Zoysia japonica* (zoysiagrass), and *Dichondra micrantha*.

[0193] Native: a “native” genetic element refers to a nucleic acid that naturally exists in, originates from, or belongs to the genome of a plant that is to be transformed. Thus, any nucleic acid, gene, polynucleotide, DNA, RNA, mRNA, or cDNA molecule that is isolated either from the genome of a plant or plant species that is to be transformed, or is isolated from a plant or species that is sexually compatible, or interfertile with the plant species that is to be transformed, is “native” to, i.e., indigenous to, the plant species. In other words, a native genetic element represents all genetic material that is accessible to plant breeders for the improvement of plants through classical plant breeding. For instance, native DNA incorporated into cultivated potato (*Solanum tuberosum*) can be derived from any genotype of *S. tuberosum* or any genotype of a wild potato species that is sexually compatible with *S. tuberosum* (e.g., *S. demissum*). Any variants of a native nucleic acid also are considered “native” in accordance with the present invention. In this respect, a “native” nucleic acid may also be isolated from a plant or sexually compatible species thereof and modified or mutated so that the resultant variant is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% similar in nucleotide sequence to the unmodified, native nucleic acid isolated from a plant. A native nucleic acid variant may also be less than about 60%, less than about 55%, or less than about 50% similar in nucleotide sequence.

[0194] A “native” nucleic acid isolated from a plant may also encode a variant of the naturally occurring protein product transcribed and translated from that nucleic acid. Thus, a native nucleic acid may encode a protein that

is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% similar in amino acid sequence to the unmodified, native protein expressed in the plant from which the nucleic acid was isolated.

[0195] *Naturally occurring nucleic acid*: this phrase means that the nucleic acid is found within the genome of a selected plant species and may be a DNA molecule or an RNA molecule. The sequence of a restriction site that is normally present in the genome of a plant species can be engineered into an exogenous DNA molecule, such as a vector or oligonucleotide, even though that restriction site was not physically isolated from that genome. Thus, the present invention permits the synthetic creation of a nucleotide sequence, such as a restriction enzyme recognition sequence, so long as that sequence is naturally occurring in the genome of the selected plant species or in a plant that is sexually compatible with the selected plant species that is to be transformed.

[0196] *Operably linked*: combining two or more molecules in such a fashion that in combination they function properly in a plant cell. For instance, a promoter is operably linked to a structural gene when the promoter controls transcription of the structural gene.

[0197] *P-DNA*: according to the present invention, P-DNA ("plant-DNA") is isolated from a plant genome and comprises at each end, or at only one end, a T-DNA border-like sequence. Thus, a P-DNA may comprise a left border-like sequence and a right border-like sequence. The border-like sequences preferably share at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 90% or at least 95%, but less than 100% sequence identity, with a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Thus, P-DNAs can be used instead of T-DNAs to transfer a desired

polynucleotide from *Agrobacterium* to a plant chromosome. The desired polynucleotide may or may not be native to the plant species to be transformed. That is, a P-DNA may be used to transfer foreign, as well as native, nucleic acids into a plant cell. Accordingly, the vectors of the present invention can be used to transfer a desired polynucleotide of the present invention (see definition above for "desired polynucleotide") into a plant genome. It is understood that all or part of the P-DNA containing the desired polynucleotide can be integrated into a plant genome by *Agrobacterium*-mediated transformation.

[0198] A P-DNA may be modified to facilitate cloning and should preferably not naturally encode proteins or parts of proteins. The P-DNA can be modified to reduce the frequency of vector backbone integration into a transformed plant genome.

[0199] A P-DNA is characterized in that it contains, at each end, at least one border sequence, referred to herein as a P-DNA "border-like" sequence, because its sequence is similar to, but not identical with, conventional T-DNA border sequences. See the definition of a "border sequence" and "border-like" above.

[0200] A desired polynucleotide and selectable marker may be positioned between the left border-like sequence and the right border-like sequence of a P-DNA of the present invention. The desired polynucleotide of the present invention and a selectable marker may comprise a gene operably linked to a variety of different nucleic acids, such as to promoter and terminator regulatory elements that facilitate their expression, i.e., transcription and/or translation of the DNA sequence encoded by the desired polynucleotide or selectable marker.

[0201] Thus, the P-DNA of the present invention may be used to transfer foreign DNA into plant genomes, as well as polynucleotides that are endogenous to plants. Accordingly, the "desired polynucleotide" that is transferred to a plant genome can be foreign, or native, or from a food-source,

and may represent a gene that is useful for producing a pharmaceutical product, such as a hormone or enzyme. The desired polynucleotide contained within the P-DNA also may be used to alter a trait associated with the transformed plant.

[0202] ***Plant tissue:*** a "plant" is any of various photosynthetic, eukaryotic, multicellular organisms of the kingdom *Plantae* characteristically producing embryos, containing chloroplasts, and having cellulose cell walls. A part of a plant, i.e., a "plant tissue" may be treated according to the methods of the present invention to produce a transgenic plant. Preferably, the plant tissue that is transformed using an *Agrobacterium*-derived vector is a germinating seedling. The inventive methods described herein, however, are not limited to the transformation of only germinating seedling. Other suitable plant tissues can be transformed according to the present invention and include, but are not limited to, pollen, leaves, stems, calli, stolons, microtubers, and shoots. Thus, the present invention envisions the transformation of angiosperm and gymnosperm plants such as turfgrass, wheat, maize, rice, barley, oat, sugar beet, potato, tomato, tobacco, alfalfa, lettuce, carrot, strawberry, cassava, sweet potato, geranium, soybean, oak, eucalyptus, walnut, and palm. According to the present invention "plant tissue" also encompasses plant cells. Plant cells include suspension cultures, callus, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, seeds and microspores. Plant tissues may be at various stages of maturity and may be grown in liquid or solid culture, or in soil or suitable media in pots, greenhouses or fields. A plant tissue also refers to any clone of such a plant, seed, progeny, propagule whether generated sexually or asexually, and descendants of any of these, such as cuttings or seed. Of particular interest are Kentucky bluegrass, creeping bentgrass, maize, and wheat, and dicots such as cotton, tomato, lettuce, *Arabidopsis*, tobacco, and geranium.

[0203] *Plant transformation and cell culture:* broadly refers to the process by which plant cells are genetically modified and transferred to an appropriate plant culture medium for maintenance, further growth, and/or further development. Such methods are well known to the skilled artisan.

[0204] *Progeny:* a “progeny” of the present invention, such as the progeny of a transgenic plant, is one that is born of, begotten by, or derived from a plant or the transgenic plant. Thus, a “progeny” plant, *i.e.*, an “F1” generation plant is an offspring or a descendant of the transgenic plant produced by the inventive methods. A progeny of a transgenic plant may contain in at least one, some, or all of its cell genomes, the desired polynucleotide that was integrated into a cell of the parent transgenic plant by the methods described herein. Thus, the desired polynucleotide is “transmitted” or “inherited” by the progeny plant. The desired polynucleotide that is so inherited in the progeny plant may reside within a P-DNA or T-DNA construct, which also is inherited by the progeny plant from its parent. The term “progeny” as used herein, also may be considered to be the offspring or descendants of a group of plants.

[0205] *Seed:* a “seed” may be regarded as a ripened plant ovule containing an embryo, and a propagative part of a plant, as a tuber or spore. Seed may be incubated prior to *Agrobacterium*-mediated transformation, in the dark, for instance, to facilitate germination. Seed also may be sterilized prior to incubation, such as by brief treatment with bleach. The resultant seedling can then be exposed to a desired strain of *Agrobacterium*.

[0206] *Seedling:* a young plant that is grown from a seed. Certain parts of a seedling, such as part or all of the scutellum may be removed prior to exposing the seedling to a solution comprising an *Agrobacterium* strain.

[0207] *Selectable/screenable marker:* a gene that, if expressed in plants or plant tissues, makes it possible to distinguish them from other plants or plant tissues that do not express that gene. Screening procedures may

require assays for expression of proteins encoded by the screenable marker gene. Examples of such markers include the beta glucuronidase (*GUS*) gene and the luciferase (*LUX*) gene. The instant invention demonstrates that cyanamide tolerance genes such as *CAH* can also be used as a marker. Thus, a gene encoding resistance to a fertilizer, antibiotic, herbicide or toxic compound can be used to identify transformation events. Examples of selectable markers include the cyanamide hydratase gene (*CAH*) streptomycin phosphotransferase (*SPT*) gene encoding streptomycin resistance, the neomycin phosphotransferase (*NPTI*) gene encoding kanamycin and geneticin resistance, the hygromycin phosphotransferase (*HPT* or *APH/V*) gene encoding resistance to hygromycin, acetolactate synthase (*als*) genes encoding resistance to sulfonylurea-type herbicides, genes (*BAR* and/or *PAT*) coding for resistance to herbicides which act to inhibit the action of glutamine synthase such as phosphinothricin (Liberty or Basta), or other similar genes known in the art.

[0208] Sequence identity: as used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified region. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences which differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage

sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

[0209] As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0210] Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981); by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85: 2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California; GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988); Higgins and

Sharp, CABIOS 5: 151-153 (1989); Corpet, et al., *Nucleic Acids Research* 16: 10881-90 (1988); Huang, et al., *Computer Applications in the Biosciences* 8: 155-65 (1992), and Pearson, et al., *Methods in Molecular Biology* 24: 307-331 (1994).

[0211] The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995); Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990); and, Altschul et al., *Nucleic Acids Res.* 25:3389-3402 (1997).

[0212] Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative

alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

[0213] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5877 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance.

[0214] BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, *Comput. Chem.*, 17:149-163 (1993)) and XNU (Claverie and States, *Comput. Chem.*, 17:191-201 (1993)) low-complexity filters can be employed alone or in combination.

[0215] Multiple alignment of the sequences can be performed using the CLUSTAL method of alignment (Higgins and Sharp (1989) *CABIOS* 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH

PENALTY=10). Default parameters for pairwise alignments using the CLUSTAL method are KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

[0216] *Trait*: a “trait” is a distinguishing feature or characteristic of a plant, which may be altered according to the present invention by integrating one or more “desired polynucleotides” and/or screenable/selectable markers into the genome of at least one plant cell of a transformed plant. The “desired polynucleotide(s)” and/or markers may confer a change in the trait of a transformed plant, by modifying any one of a number of genetic, molecular, biochemical, physiological, morphological, or agronomic characteristics or properties of the transformed plant cell or plant as a whole. Thus, expression of one or more, stably integrated desired polynucleotide(s) in a plant genome, may alter a trait that is selected from the group consisting of, but not limited to, increased drought tolerance, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved vigor, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, and improved flower longevity.

[0217] *Transcription and translation terminators*: The expression vectors of the present invention typically have a transcriptional termination region at the opposite end from the transcription initiation regulatory region. The transcriptional termination region may be selected, for stability of the mRNA to enhance expression and/or for the addition of polyadenylation tails added to the gene transcription product.

[0218] *Transfer DNA (T-DNA)*: an *Agrobacterium* T-DNA is a genetic element that is well-known as an element capable of integrating a nucleotide sequence contained within its borders into another nucleotide. In

this respect, a T-DNA is flanked, typically, by two “border” sequences. A desired polynucleotide of the present invention and a selectable marker may be positioned between the left border-like sequence and the right border-like sequence of a T-DNA. The desired polynucleotide and selectable marker contained within the T-DNA may be operably linked to a variety of different, plant-specific (*i.e.*, native), or foreign nucleic acids, like promoter and terminator regulatory elements that facilitate its expression, *i.e.*, transcription and/or translation of the DNA sequence encoded by the desired polynucleotide or selectable marker.

[0219] Transformation of plant cells: A process by which a nucleic acid is stably inserted into the genome of a plant cell. Transformation may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of nucleic acid sequences into a prokaryotic or eukaryotic host cell, including *Agrobacterium*-mediated transformation protocols, viral infection, whiskers, electroporation, microinjection, polyethylene glycol-treatment, heat shock, lipofection and particle bombardment.

[0220] Transgenic plant: a transgenic plant of the present invention is one that comprises at least one cell genome in which an exogenous nucleic acid has been stably integrated. According to the present invention, a transgenic plant is a plant that comprises only one genetically modified cell and cell genome, or is a plant that comprises some genetically modified cells, or is a plant in which all of the cells are genetically modified. A transgenic plant of the present invention may be one that comprises expression of the desired polynucleotide, *i.e.*, the exogenous nucleic acid, in only certain parts of the plant. Thus, a transgenic plant may contain only genetically modified cells in certain parts of its structure.

[0221] Undesirable DNA: any DNA that is not derived from a common food source and is not essential for expression of a beneficial trait in a transgenic plant, when making a genetically engineered food crop. Under

these circumstances, undesirable DNA is DNA from viruses, bacteria, fungi, animals, and non-edible plants.

[0222] Vortexing, turbo-vortexing: either term refers to the abrupt agitation of plant tissues, such as germinating seedling, using a standard vortex or other device. According to the present invention, plant tissues may be vortexed from 60 seconds to several hours. Preferably, the plant tissue is vortexed for about 5 to about 30 minutes. It is well within the purview of the skilled artisan to determine a suitable length of time to vortex plant tissues from various monocotyledon and dicotyledon plant species.

[0223] Variant: a “variant,” as used herein, is understood to mean a nucleotide or amino acid sequence that deviates from the standard, or given, nucleotide or amino acid sequence of a particular gene or protein. The terms, “isoform,” “isotype,” and “analog” also refer to “variant” forms of a nucleotide or an amino acid sequence. An amino acid sequence that is altered by the addition, removal or substitution of one or more amino acids, or a change in nucleotide sequence, may be considered a “variant” sequence. The variant may have “conservative” changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. A variant may have “nonconservative” changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted may be found using computer programs well known in the art such as Vector NTI Suite (InforMax, MD) software. “Variant” may also refer to a “shuffled gene” such as those described in Maxygen-assigned patents. For instance, a variant of the present invention may include variants of sequences and desired polynucleotides that are modified according to the methods and rationale disclosed in U.S. 6,132,970, which is incorporated herein by reference.

[0224] It is understood that the present invention is not limited to the particular methodology, protocols, vectors, and reagents, etc., described

herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a gene" is a reference to one or more genes and includes equivalents thereof known to those skilled in the art and so forth. Indeed, one skilled in the art can use the methods described herein to express any native gene (known presently or subsequently) in plant host systems.

[0225] A surprising discovery of the present invention is that a germinating seedling that is agitated in a solution containing *Agrobacterium* cells harboring a vector that contains a desired polynucleotide can be planted into soil according to the methods described herein, and grown into a plant that contains cells that are stably transformed with the desired polynucleotide. Accordingly, the first, most basic method of the present invention entails vortexing germinating seedling with an *Agrobacterium* strain containing an appropriate vector, and then simply planting the vortexed seedling in soil, under conditions that promote growth.

[0226] The efficiency of stable transformation can be further enhanced by inducing double strand breaks in the chromosomes of germinating seedling before, during, and/or after infection. Such double strand breaks can be generated by, for instance, subjecting seedlings to low doses of chemicals such as methyl methane sulfonate (MMS), HO-endonuclease, bleomycin, neocarzinostatin, camptothecan, and cisplatin, or by using ionizing radiation or heavy ions. Similar effects may also be accomplished by temporarily blocking the cell's own double strand gap repair mechanism. Mutations that may inadvertently arise from these treatments can be easily removed by back-crossing transgenic plants with untransformed plants.

[0227] The efficiency of *Agrobacterium*-mediated transformation also can be enhanced by exposing the plant/transformation sample to (i) a purine inhibitor or (ii) a purine inhibitor and a pyrimidine inhibitor. Roberts *et al.*, PNAS, vol. 100 (11), pp 6634-6639, reported that disruption of purine synthesis in host cells induces "supersensitivity" to *A. tumefaciens* transformation, and that inhibitors that blocked both purine and pyrimidine synthesis had an even greater enhancing effect on transformation efficiency than a purine inhibitor alone.

[0228] To that end, the present invention contemplates exposing a plant sample to one or more purine inhibitors, or to a mixture of purine and pyrimidine inhibitors, or to a substance that is both a purine inhibitor and a pyrimidine inhibitor in conjunction with the methods described herein. Examples of purine inhibitors include mizoribine, azathioprine, mycophenolic acid, methotrexate, and mycophenolate mofetil. Examples of pyrimidine inhibitors include 5-fluorouracil, Brequinar sodium, and Leflunomide. Examples of purine synthesis- and pyrimidine- inhibitors include azaserine, acivicin, methotrexate and its polyglutamate derivatives, and cyclophosphamide.

[0229] Thus, the present invention encompasses adding, exposing, or incubating a plant tissue to an agent before, during, or after practicing the inventive agitation-transformation method, to an agent that enhances transformation efficiency, wherein the agent is a purine-, pyrimidine-, or purine- and pyrimidine- inhibitor. The plant tissue could be exposed to the agent for a short period of time, for example, only during the agitation step, or only briefly prior to agitation.

[0230] The concentration of the agent to which the plant tissue is exposed may be at least 1 $\mu\text{g}/\text{ml}$, 2 $\mu\text{g}/\text{ml}$, 3 $\mu\text{g}/\text{ml}$, 4 $\mu\text{g}/\text{ml}$, 5 $\mu\text{g}/\text{ml}$, 6 $\mu\text{g}/\text{ml}$, 7 $\mu\text{g}/\text{ml}$, 8 $\mu\text{g}/\text{ml}$, 9 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$, 11 $\mu\text{g}/\text{ml}$, 12 $\mu\text{g}/\text{ml}$, 13 $\mu\text{g}/\text{ml}$, 14 $\mu\text{g}/\text{ml}$, 15 $\mu\text{g}/\text{ml}$, 16 $\mu\text{g}/\text{ml}$, 17 $\mu\text{g}/\text{ml}$, 18 $\mu\text{g}/\text{ml}$, 19 $\mu\text{g}/\text{ml}$, 20 $\mu\text{g}/\text{ml}$, 21 $\mu\text{g}/\text{ml}$, 22 $\mu\text{g}/\text{ml}$, 23 $\mu\text{g}/\text{ml}$, 24 $\mu\text{g}/\text{ml}$, 25 $\mu\text{g}/\text{ml}$, 26 $\mu\text{g}/\text{ml}$, 27 $\mu\text{g}/\text{ml}$, 28 $\mu\text{g}/\text{ml}$, 29 $\mu\text{g}/\text{ml}$, 30

µg/ml, 31 µg/ml, 32 µg/ml, 33 µg/ml, 34 µg/ml, 35 µg/ml, 36 µg/ml, 37 µg/ml, 38 µg/ml, 39 µg/ml, 40 µg/ml, 41 µg/ml, 42 µg/ml, 43 µg/ml, 44 µg/ml, 45 µg/ml, 46 µg/ml, 47 µg/ml, 48 µg/ml, 49 µg/ml, 50 µg/ml, 51 µg/ml, 52 µg/ml, 53 µg/ml, 54 µg/ml, 55 µg/ml, 56 µg/ml, 57 µg/ml, 58 µg/ml, 59 µg/ml, 60 µg/ml, 61 µg/ml, 62 µg/ml, 63 µg/ml, 64 µg/ml, 65 µg/ml, 66 µg/ml, 67 µg/ml, 68 µg/ml, 69 µg/ml, 70 µg/ml, 71 µg/ml, 72 µg/ml, 73 µg/ml, 74 µg/ml, 75 µg/ml, 76 µg/ml, 77 µg/ml, 78 µg/ml, 79 µg/ml, 80 µg/ml, 81 µg/ml, 82 µg/ml, 83 µg/ml, 84 µg/ml, 85 µg/ml, 86 µg/ml, 87 µg/ml, 88 µg/ml, 89 µg/ml, 90 µg/ml, 91 µg/ml, 92 µg/ml, 93 µg/ml, 94 µg/ml, 95 µg/ml, 96 µg/ml, 97 µg/ml, 98 µg/ml, 99 µg/ml, or 100 µg/ml, or any range in between.

[0231] Also contemplated in the present invention are new cyanamide resistance genes, especially those depicted in SEQ ID NOs. 12, 14, or 15, which each encode a cyanamide resistance protein that comprises the amino acid sequence depicted in SEQ ID NO. 13.

[0232] The cyanamide resistance gene that was isolated from *Aspergillus terricola* is depicted in SEQ ID NO. 12. The genes depicted in SEQ ID NOs. 14 and 15 represent codon-optimized nucleotide sequences of SEQ ID NO. 12. That is, SEQ ID NO. 14 is a cyanamide resistance gene that has been codon optimized so as to enhance expression of the cyanamide resistance protein in monocotyledonous plants; while SEQ ID NO. 15 is a cyanamide resistance gene that has been codon optimized so as to enhance expression of the cyanamide resistance protein in dicotyledonous plants. Nevertheless, any of the cyanamide resistance genes depicted in SEQ ID NOs 12, 14, or 15 can be used to confer cyanamide resistance in any type of plant.

[0233] Accordingly, the inventive methodology may entail vortexing a plant tissue with an *Agrobacterium* vector to optimize transfer of the vector and desired polynucleotide(s) into plant cells, and also the induction of double stranded breaks in plant chromosomes to increase the frequency of stably

transforming, i.e., integrating, the plant genome with the desired polynucleotide(s).

[0234] The transgenic plant is crossed or self-fertilized to transmit the desired gene or nucleotide sequence to progeny plants. Seedlings of this next generation of transgenic plants can be screened for the presence of a desired polynucleotide using standard techniques such as PCR, enzyme or phenotypic assays, ELISA, or Western blot analysis. Alternatively, if the transformation vector comprises a selectable/screenable marker(s), the plant progeny may be selected for resistance or tolerance to a particular substance, as is described in detail below. While vortexing is a preferred method of exposing plant tissues to *Agrobacterium* strains, the present invention is not limited to such a method.

[0235] The second method entails transferring the *Agrobacterium*-transformed seedling to soil only after the seedling has been nurtured on minimal tissue culture medium (e.g. MS - Murashige & Skoog, *Physiol. Plant.*, 15: 473-479, 1962), without the induction of a callus. The "pre-planting" nurturing step helps to boost the strength, nutrients, and resources available to the seedling prior to planting directly in soil.

[0236] The third inventive method encompasses inducing the transformed seedling to undergo a callus phase, stimulating the growth of shoots and roots, and then planting directly in soil. To perform the latter, the present invention provides a novel *Agrobacterium* transformation vector, that may, or may not, be used in conjunction with the novel vortex method for transforming seedlings.

[0237] The novel transformation vector of the present invention comprises an alternative to the *Agrobacterium*-derived T-DNA element , which is characterized by a "left border" at its 5'-end, and a "right border" at its 3'-end. According to the invention, the alternative transfer DNA may be isolated from an edible plant in order to minimize the quantity of undesirable nucleic

acids introduced into the target plant genome. Such a plant transfer DNA (P-DNA) also is delineated by left and right border-like sequences that support the transfer of one polynucleotide into another. For the purposes of the present invention, either T-DNA or P-DNA constructs can be used to transfer a desired polynucleotide into a plant cell. The skilled artisan would understand that, in some instances, it is desirable to reduce the amount and number of undesirable genetic elements that are introduced into a plant genome via *Agrobacterium*-mediated transformation. Accordingly, the skilled artisan could use the P-DNA of the present invention in such instances, because the P-DNA, and its border-like sequences, is isolated from a plant genome.

[0238] According to the present invention, a desired polynucleotide is positioned within such a P-DNA or T-DNA and is operably linked to a promoter and a terminator, that can express it. In order to further minimize the quantity of foreign nucleic acid introduced into a plant genome after successful transformation, the promoter and terminator linked to the desired polynucleotide may be promoters and terminators that naturally occur in a plant genome.

[0239] If required, a selectable marker that confers a detectable trait to plant cells containing it, can be positioned within the T-DNA/P-DNA of the inventive vector. Such a selectable marker may encode proteins that confer tolerance to herbicides such as glyphosate-N-acetyltransferase (*GAT*) or 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*). A preferred selectable marker gene confers antibiotic resistance to transgenic plants, such as the neomycin phosphotransferase gene. Another preferred selectable marker gene provides cyanamide tolerance. One example of a cyanamide tolerance gene is the *Myrothecium verrucaria* cyanamide hydratase (*CAH*) gene. The instant invention demonstrates that distant homologs of the *CAH* gene, derived from soil fungi such as *Aspergillus*, *Cladosporium*, and *Penicillium* (but not from the yeast species *Saccharomyces cerevisiae*) also function as cyanamide tolerance genes.

[0240] Calcium cyanamide is an environment-friendly nitrogen fertilizer. Because nitrogen is released only gradually, it poses less risk of nitrate pollution to groundwater than do the popular urea-based or ammonium-nitrate-based fertilizers. Furthermore, it provides beneficial additional effects because both the lime and cyanamide breakdown products such as dicyandiamide limit growth of undesirable fungi and parasites including *Sclerotinia*, *Pythium*, *Erysiphe* and nematodes, whereas it stimulates growth of the beneficial fungi *Aspergillus* and *Penicillium*.

[0241] One reason calcium cyanamide is not widely used in agriculture is that it can only be applied pre-emergence. However, tolerance to cyanamide makes it possible now to apply cyanamide during and after emergence. By using cyanamide-tolerant transgenic plants, calcium cyanamide can be applied both as a pre- and post-emergence fertilizer to increase yield and quality of crops and other agronomically important plants.

[0242] Thus, the present invention provides a novel combination of cyanamide fertilizer and cyanamide-tolerant plants to reduce the prevalence of soil-borne fungi, nematodes and insects, thereby increasing crop yield and quality. Enhanced disease and pest control can be obtained by not only applying before emergence but also during the growth phase of the plant.

[0243] The post-emergence application of calcium cyanamide is also predicted to limit the growth of undesirable plants, such as weeds, that are not naturally cyanamide tolerant. Such an application would limit the growth of multiple weeds including annual bluegrass, goosegrass, crowfootgrass, dollarweed, purple nutsedge, torpedograss, kyllinga, and alligatorweed on lawns planted with cyanamide-tolerant turf grass.

[0244] The present invention eliminates the need for explant starting material, such as immature plant embryos. Thus the inventive methodology is species-independent, cost-effective, and less labor intensive, than

conventional species-dependent methods that require selection, proliferation, and regeneration of individually transformed somatic cells.

Seedling characteristics

[0245] The inventive methodology utilizes a seedling that has only just begun to germinate and which is characterized, in a monocotyledonous or dicotyledonous plant, by a just-emerging coleoptile or cotyledon at the surface of the seed coat.

[0246] There may be an optimal stage of cotyledon emergence, i.e., germination, in seeds that provides a high frequency of transformation. For tobacco seeds, for instance, a high level of transformation frequency via agitation is observed when the cotyledon is one-half to three-quarters emerged from the seed coat. The time it takes to establish the optimal cotyledon emergence stage will vary depending on the specific dicotyledon species and the environmental conditions during germination, such as light, moisture, temperature, and the emergence medium (soil, artificial medium, sand, etc.).

[0247] One skilled in the art would know how to systematically define these environmental parameters for each dicotyledon seed species in order to determine the optimal cotyledon emergence stage. In this fashion, one may optimize when to agitate a germinating seed so as to obtain a high frequency of transformation. One may quantify the level of transformation by monitoring transient GUS expression assays or by stable transformation. For monocotyledon plants, such as turf and wheat, one would develop a timing of transformation based upon optimal coleoptile emergence instead of cotyledon emergence.

[0248] A seedling that is at such an early-stage of germination will possess cells that are rapidly proliferating as the seed develops. Furthermore, certain cells of the coleoptile may be progenitors of germ line cells, which means that transforming these cells in particular will increase the

likelihood of obtaining an inheritable, but artificial or modified, trait.

Accordingly, the present invention makes use of this naturally-occurring state of cell multiplication and development by exposing these seedlings to an *Agrobacterium* vector that contains a gene or nucleotide sequence that the skilled artisan wishes to integrate into cells of the germinating seedling.

Agitation

[0249] In particular, a seedling that is characterized by a just-emerging coleoptile or cotyledon may be agitated in a solution that contains an *Agrobacterium* strain. For instance, such a seed may be placed into a tube or some other vessel that contains an *Agrobacterium* solution, which is then vortexed in a standard bench-top vortex for a short period of time. A tube containing a seedling in solution may be turbo-vortexed. Alternatively, the seedling may be submerged into a solution that is mixed for some period of time with a magnetic stir-bar using a standard bench-top mixing device.

Vortexing

[0250] The vortexing step described above may be enhanced by adding a small amount of sand to the *Agrobacterium*-containing solution. In experiments with tobacco and geranium, for example, the inclusion of a small amount of sand in the transfection solution during vortexing greatly increased the frequency of transformation. Other materials in place of sand that act in an abrasive fashion may be added to the *Agrobacterium*-containing transfection solution, such as, but not limited to, small glass beads, silicon, plastic grains, or stone. Turbo-vortexing also may be employed to facilitate transformation.

[0251] Depending on the size of the germinating seedling and the intensity of the agitation, different seedlings from different plant species, may be vortexed for different periods of time, such as anywhere from a few seconds, or 1-15 minutes, 5-10 minutes, 1-5 minutes, 15-20 minutes, an hour, or several hours. Small germinating seedlings from plants such as tobacco,

turfgrass and *Arabidopsis*, for instance, may require less agitation than larger germinating seedlings such as wheat, maize and cotton.

Removing plants that comprise vector backbone sequences

[0252] It is possible that DNA from the vector portion flanking the P-DNA or T-DNA of a transformation construct is incorporated into the host plant genome while agitating a germinating seed in the *Agrobacterium*-containing transformation solution. Thus, it is necessary to distinguish plants that contain only the desired polynucleotide insert integrated into their genome and from plants that also contain regions of the plasmid vector (*i.e.*, “backbone DNA”) after transformation. Backbone DNA is the part of an *Agrobacterium* binary vector that excludes the T-DNA/P-DNA.

[0253] In order to facilitate identification of plants that contain backbone DNA, a “backbone integration marker,” which alters some morphological feature of the plant, is placed upstream and/or downstream of the T-DNA/P-DNA. Thus, it is possible a backbone integration marker gene that changes the shape of the transformed plant’s leaves, roots, stem, height or some other morphological feature, that is not attributable to an effect of the desired polynucleotide, can be used to identify plants that contain vector backbone sequences. The color, texture or other traits of a plant may be similarly altered. “Morphological” refers to the form and structure of an organism without particular consideration of function, or which relates directly to the form and structure of an organism or its parts.

[0254] Thus, a transformed plant that has a morphologically altered feature as compared to a non-transformed or wild-type plant of that plant species, is indicative of a plant that contains backbone vector DNA in its genome.

[0255] Accordingly, an *Agrobacterium* vector may also carry an operable cytokinin gene upstream and/or downstream of the insertion DNA that will alter some morphological feature of the plant if it is integrated into the

plant genome. Thus, it is straightforward to distinguish between desired and undesired transformation events. Transformed plants that exhibit such an altered morphological feature can be removed from the pool of desired plants, because they must contain undesirable, i.e., backbone, DNA sequences integrated into the genome. In this way, plant genomes that contain integrated and undesirable vector sequences, as well as an integrated desired polynucleotide, can be identified by detecting the expression of the cytokinin gene. Thus, transgenic plants produced by the method of the present invention that display a cytokinin-overproducing phenotype can be discarded, while those that are indistinguishable from untransformed plants can be maintained for further analysis. A preferred cytokinin gene is the *Agrobacterium* isopentenyl phosphotransferase (*IPT*) gene. Another cytokinin gene is, for instance, the *Agrobacterium* transzeatine synthase (*TZS*) gene. The present invention is not limited to the use of only a cytokinin gene. Any gene that alters a morphological feature of a plant can be used similarly.

[0256] Another strategy for identifying plants stably transformed with only desired DNA is to PCR amplify genomic DNA prepared from the plant using combination of primer pairs designed to the desired and to backbone vector DNA sequences. Genomes from plants that produce PCR products using primers designed to the backbone vector sequences are from plants that contain integrated backbone DNA.

[0257] Thus, by either using the expression of a gene to change a morphological feature of a plant, or by screening for stably integrated foreign DNA in a transformed plant, plants stably transformed with only desired DNA sequences can be identified and selected.

[0258] Similarly, while the stable integration of marker genes into the genomes of plant cells facilitates the identification of transformation events, such modifications of plant genomes are undesirable because marker genes usually represent foreign DNA that can be harmful to the plant, and to

elements in the surrounding environment. Use of a marker gene can be avoided through modification of conventional *Agrobacterium*-based methods.

[0259] It is known that plant cells exposed during agitation to two different *Agrobacterium* strains, can receive T-DNAs from both strains. One of the *Agrobacterium* strains used for plant infection may contain a mutant *virD2* gene. This mutant *Agrobacterium* strain is capable of transferring T-DNAs to plant nuclei but most of these T-DNAs will fail to integrate into the plant genome (Shurvinton *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 89: 11837-11841, 1992; Mysore *et al.*, *Mol. Plant Microbe Interact.*, 11: 668-683, 1998). The mutant *Agrobacterium* strain can further contain a marker gene such as the neomycin phosphotransferase (*NPTII*) gene, operably linked to a promoter and followed by a termination signal, between T-DNA borders. Infection of explants with this mutant strain will result in temporary marker gene expression in some plant cells. Only plant cells that transiently express the marker gene are able to survive media that contain a selection agent such as kanamycin.

[0260] The virulent *Agrobacterium* strain that contains a wild-type *virD2* gene carries the recombinant DNA molecule of interest but lacks a marker gene. Upon co-infection, some plant cells will contain both a non-integrating T-DNA with the marker gene and an integrating carrier DNA with the sequences of interest. In fact, 65% of tobacco cells containing at least one T-DNA derived from one of the strains have been shown to also contain at least one T-DNA from the other strain (De Neve *et al.*, *Plant J.*, 11:15-29, 1997; De Buck *et al.*, *Mol. Plant Microbe Interact.*, 11: 449-57, 1998).

[0261] After about 5 to 10 days, the infected seedlings or explants are transferred to media lacking the selection agent to support further growth of events that had survived the temporary selection period. A significant percentage of these events contain the T-DNA carrying a recombinant DNA molecule of interest and lack the T-DNA with a selectable marker gene for transformation.

[0262] *Agrobacterium* strains that contain a functional *virD2* gene instead of mutant *virD2* for transient marker gene expression may also be used for selection of plant transformants. However, the frequency of obtaining genetically modified plants lacking a marker gene is generally low compared to use of the mutant *virD2* gene.

[0263] Cells that transiently express a marker gene can be discriminated from cells that don't express such a gene using a variety of selection systems. However, not all these selection systems are equally suitable. In potato and tobacco, the most preferred selection agents are kanamycin (about 100 mg/L) and paramomycin (about 25-50 mg/L) because they arrest untransformed cells within 5 to 10 days. Other selection agents include hygromycin, glyphosate, glufosinate and cyanamide. The marker genes corresponding to these various agents encode neomycin phosphotransferase (*NPTII*) for kanamycin or paramomycin resistance, hygromycin phosphotransferase (*HPTII*) for resistance to hygromycin, 5-enolpyruvul-3-phosphoshikimic acid synthase (*EPSPS*) for glyphosate resistance, phosphinothricin acetyltransferase (*PAT*) for glufosinate resistance, and cyanamide hydratase (*CAH*) for cyanamide resistance.

[0264] An alternative way to develop transgenic plants lacking a selectable marker gene is based on excision of the marker gene cassette after plant transformation. Such excision can be accomplished by, e.g., placing a constitutively expressed marker gene together with an inducible *Cre* gene between two *lox* sites. Induction of the *Cre* gene would then in certain cases result in excision of all sequences between the *lox* sites. One example of an inducible promoter is the sunflower Ha hsp17.7 G4 promoter (Coca *et al.*, *Plant Mol. Biol.*, 31: 863-76, 1996). By subjecting regenerating plantlets to a mild heat shock, induction of the heat shock promoter will lead to *Cre* gene expression and subsequent ejection of the region between the *lox* sites in some of the transformants.

[0265] The present invention contemplates the integration, for example, of any desired polynucleotide into a cell of a plant using the inventive methods. Particularly preferred desired polynucleotides of the present invention that can be integrated into a plant genome and expressed according to the methodologies described herein, include, but are not limited to, (i) the synthetic peptide gene D4E1 (U.S. Patent 6,084,156; U.S. Patent 6,018,102) to confer bacterial resistance to transgenic plants such as geranium; (ii) the HOS1 gene homologs to enhance cold, freezing and salt tolerance in transgenic plants through gene silencing (Lee *et al.*, *Gene and Develop.*, 15: 912-924, 2001); (iii) the *Vitreoscilla* hemoglobin gene (U.S. Patent 5,959,187) to develop greener and insect tolerant turfgrass that displays increased seed germination and enhanced vigor; and (iv) genes involved in the lignin biosynthetic pathway.

[0266] Other plant traits whose expression can be modified, introduced, reduced, or increased by integrating a foreign or native desired polynucleotide or variant thereof into a plant genome by the inventive methodology, include traits selected from the group consisting of, but not limited to, increased drought tolerance, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved vigor, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, and improved flower longevity.

[0267] The examples below are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may be used.

EXAMPLES

Example 1

Development of a species-independent method to obtain transgenic plants without the need for plant cell proliferation and regeneration

[0268] Binary vectors that were created to develop a species-independent transformation method carry an intron-containing beta glucuronidase (*GUS*) gene (Genbank accession number AF354045) operably linked to a promoter and terminator. The MMV24P promoter of *mirabilis* mosaic virus (Maiti et al., US Patent 6420547, 2002), and the promoter of the sugarcane ubiquitin-4 gene (Albert and Wei, US Patent 20020046415A1, 2002) were used to transform dicotyledonous and monocotyledonous plants, respectively. The binary vectors were introduced into *Agrobacterium* by incubating competent LBA4404 cells (50 µL) with 1 µg of vector DNA for 5 minutes at 37°C, freezing for about 15 seconds in liquid nitrogen (about –196°C), and incubating again at 37°C for 5 minutes. After adding 1 mL of liquid broth (LB), the treated cells were grown for 3 hours at 28°C and plated on LB/agar containing streptomycin (100 mg/L) and kanamycin (100 mg/L). The vector DNAs were then isolated from overnight cultures of individual LBA4404 colonies and examined by restriction analysis to confirm their integrity.

[0269] The resulting *Agrobacterium* strains were used to successfully transform eight different plant systems.

1. *Arabidopsis thaliana*

[0270] First, seed of the *Arabidopsis thaliana* ecotype Columbia was sterilized by turbo-vortexing with 20% bleach. The sterile seed was then incubated for 2 days at room temperature in the dark to allow germination. The germinating seedlings were then emerged into an *Agrobacterium* suspension, which was obtained by resuspending precipitated cells of an

overnight-grown culture in MS medium to obtain an optical density of 0.6 – 0.75. The mixture was turbo-vortexed using a high-performance microcentrifuge tube attachment for the Vortex-Genie 2 Mixer (Part # SI-0563) manufactured by Scientific Industries, Inc., Airport Orville Drive, Bohemia, NY 11716 at a speed setting of "4" for 5 to 30 minutes. The treated seedlings were transferred to either soil or MS medium not containing any hormones, and incubated at 25°C. After 3 weeks, plants were sampled to assay for GUS expression (Jefferson et al., *EMBO J* 6: 3901-3907, 1987). Approximately 13% of tested plants (168 of 1274) displayed a blue color in significant portions of both petioles and leaves (Table 2). GUS assays on control plants that had been infected without vortexing were negative. A total of 10 randomly chosen GUS-positive plants were grown for 4 more weeks at 25°C to allow seed set. The resulting seed was sterilized and germinated on MS medium, and progenies were then GUS assayed to determine the frequency of transgene transmission to the next generation. These analyses demonstrated that up to 78% of progeny plants represented stably transformed lines (Table 3).

2. *Nicotiana tabacum* (tobacco)

[0271] Second, seed of the *Nicotiana tabacum* (tobacco) variety SR-1 was sterilized by turbo-vortexing with 20% bleach. The sterile seed was then incubated for 5 days at room temperature in the dark to allow germination. Seedlings were turbo-vortexed with *Agrobacterium* as described above. After 2 days of co-cultivation, the treated seedlings were transferred to either soil or MS medium not containing any hormones, and incubated for about 3 weeks at 25°C. Treated seedlings were then assayed for GUS expression. As shown in Table 4, a 5-minute vortex-period resulted in a frequency of GUS-expressing seedlings of approximately 7% (44 of 628 seedlings); a 30-minute vortex-period resulted in a slightly lower efficiency (Table 4). Four randomly chosen GUS-positive seedlings were grown for 12 more weeks at 25°C to allow seed set. The resulting seed was sterilized and

germinated on MS medium, and progenies were then GUS assayed to determine the frequency of transgene transmission to the next generation. To confirm the presence of the GUS gene, DNA was extracted from T1 seedlings and used to perform a PCR analysis. These phenotypic and molecular analyses demonstrated that 21% of the progeny plants represented stably transformed lines (Table 5).

3. *Gossypium hirsutum* (cotton)

[0272] Third, seed of the *Gossypium hirsutum* (cotton) variety Coker-312 was sterilized by turbo-vortexing with 20% bleach. After removal of seed coat and cotyledons, the sterile seed was incubated for 2 days at room temperature in the dark to allow germination. Seedlings were then Agro-infected in a similar way as described above, except that turbo-mixing was carried out for 15 minutes. The treated seedlings were transferred to MS medium not containing any hormones, and incubated at 25°C. After 3 weeks, samples of individual seedlings were assayed for GUS expression. A very high percentage of these leaves (50%) developed an intense blue color in stems, petioles and leaves, indicating that a high proportion of cells stably expressed the GUS gene. These seedlings are allowed to grow into mature plants and set seed. The frequency of transformation events that is transmitted to the next generation can be determined by screening progeny plants for GUS expression. Approximately 5-75% of progeny plants is predicted to represent stably transformed lines.

4. *Lactuca sativa* (lettuce)

[0273] Fourth, seed of the *Lactuca sativa* (lettuce) variety "Royal Oak Leaf" was sterilized, germinated for 3 days, and turbo-vortexed with *Agrobacterium* as described above. After 2 days of co-cultivation, the treated seedlings were transferred to MS medium not containing any hormones, and incubated for about 3 weeks at 25°C. Treated seedlings were then assayed for GUS expression. Seventy percent of lettuce seedlings displayed GUS

activity, demonstrating that the marker-free transformation method is particularly effective in this crop system. About 5-75% of progeny plants are expected to contain a transmitted transgene.

5. *Lycopersicon esculentum* (tomato)

[0274] Fifth, seed of the *Lycopersicon esculentum* (tomato) variety variety "Juliet hybrid" was sterilized, germinated for 4 days, and turbo-vortexed with *Agrobacterium* as described above. After 2 days of co-cultivation, the treated seedlings were transferred to MS medium not containing any hormones, and incubated for about 3 weeks at 25°C. Treated seedlings were then assayed for GUS expression. Ninety percent of tomato seedlings displayed GUS activity, demonstrating that the marker-free transformation method is particularly effective in this crop system. About 5-75% of progeny plants are expected to contain a transmitted transgene.

6. *Agrostis palustris* (creeping bentgrass)

[0275] Sixth, seed of the *Agrostis palustris* (creeping bentgrass) variety L-93 was sterilized by turbo-vortexing with 20% bleach. The sterile seed was incubated at room temperature in the dark to allow germination. After 1 week, the germinating seedlings were turbo-vortexed with *Agrobacterium* for approximately 30 minutes. The infected seedlings were transferred to either soil or MS medium not containing any hormones, and incubated at 25°C. At several time points, the seedlings were assayed for GUS expression . Three days post-infection, all seedlings displayed a uniformly blue color in all tissues, indicating that the GUS gene was transferred effectively to the nuclei of a large proportion of plant cells. Even after 3 weeks, a high frequency of seedlings (22 of 106) still displayed a blue color in all tissues, indicating that most or all the cells of these seedlings contained the GUS gene stably integrated in their genomes. The frequency of seedlings that developed at least some blue color at the latter time point was 35% (37 of 106). This experiment was repeated several times with similar

results. Seedlings that tested positive for uniform GUS expression were grown for an additional three weeks and subsequently transferred to a vernalization chamber set at 2°C. After a 2-month incubation period, the plants can be transferred to another growth chamber, and grown for 2 months at 25°C with a 16-hour photoperiod to allow flowering and seed set. The harvested progeny seed can be planted in soil, and 2-week old plants can be PCR analyzed for the presence of the *GUS* gene. Approximately 5-75% of progeny plants derived from *GUS*-positive T0 plants is predicted to contain the transmitted *GUS* gene.

7. *Triticum aestivum* (wheat)

[0276] Seventh, seed of the *Triticum aestivum* (wheat) variety "Bobwhite" was sterilized by vortexing with 20% bleach. The sterile seed was incubated for 2 days at room temperature in the dark to allow germination. After removal of the scutellum, seedlings were turbo-vortexed with an *Agrobacterium* strain carrying the GUS vector. Surprisingly, these treated seedlings only comprising coleoptile and coleorhiza developed vigorously on MS medium not containing any hormones, and could be transferred to soil within three weeks. Almost all seedlings displayed a blue color after three days, indicating transient *GUS* gene expression. Approximately 4.5% of leaves still displayed large blue sectors on leaves and petioles, even after 3 weeks, indicating that many cells of these leaves contained the *GUS* gene stably integrated into their genomes. This experiment was repeated with similar results. *GUS*-positive seedlings were allowed to grow into mature plants and flower. DNA extracted from these flowers confirmed the presence of the *GUS* gene in at least some of the flower cells. Approximately 5-75% of progeny plants derived from *GUS*-positive flowers is predicted to contain the transmitted *GUS* gene.

8. Z. a mays (maize)

[0277] Eighth, seed of the recalcitrant *Zea mays* (maize) variety "Bonus" was sterilized by vortexing with 20% bleach. The sterile seed was incubated for 2 days at room temperature in the dark to allow germination. After removal of the scutellum, seedlings were infected with an *Agrobacterium* strain carrying pSIM115 or similar vectors. The treated seedlings were transferred to MS medium not containing any hormones, and incubated at 25°C. Of all seedlings transiently expressing the *GUS* gene three days after infection, about 5.5% still displayed an intense blue color 3 weeks later. Thus, a relatively high proportion of transferred DNAs succeeded in stably integrating into the plant genome. GUS-positive seedlings were transferred to the greenhouse, and are allowed to grow into flowering plants. PCR analysis is expected to confirm the presence of the *GUS* gene in about 5% of the flowers. Approximately 5-75% of progenies derived from these flowers are predicted to represent transgenic events.

9. Medicago sativa (alfalfa)

[0278] Ninth, seed of the *Medicago sativa* (alfalfa) variety variety "FG 40M157" was sterilized, germinated for 3 days, and turbo-vortexed with *Agrobacterium* as described above. After 2 days of co-cultivation, the treated seedlings were transferred to MS medium not containing any hormones, and incubated for about 5 weeks at 24°C. Treated seedlings (81) were then assayed for GUS expression. Eighty-nine percent of alfalfa seedlings displayed GUS activity, demonstrating that the marker-free transformation method is particularly effective in this crop system. About 5-75% of progeny plants are expected to contain a transmitted transgene.

[0279] The above experiments demonstrate that vortex-mediated seedling transformation is an effective and generally-applicable method to generate transgenic monocotyledonous and dicotyledonous plants. Transgenic plants developed through this species-independent method do not contain undesirable marker genes.

Example 2

Optimized integration of transferred DNAs

[0280] Example 1 demonstrates that the transfer of DNA from *Agrobacterium* to individual plant cell nuclei can be optimized for many different plant species by agitating seedlings in *Agrobacterium* suspensions. This example also shows that not all the transferred DNAs subsequently integrate into the plant cell genome. To optimize the second phase of the transformation process, 100 maize seedlings were infected as described in Example 1, and placed on media that contain low levels (50 parts per million) of methyl methane sulfonate (MMS), from 1 day prior to infection until 1 day after infection. An additional 100 seedlings were placed on control media that lack MMS. Approximately 2 weeks after infection, seedlings were assayed for stable GUS expression. Interestingly, 25% of MMS-treated seedlings contained multiple blue sectors on all assayed tissues whereas only 2.5% of control seedlings contained an occasional blue spot. Thus, the frequency of stable transformation can be increased at least 12.5-fold by using agents that trigger double strand breaks.

[0281] This experiment was repeated two times with higher concentrations of MMS, to determine whether integration efficiencies could be further enhanced. Maize seedlings were subjected to media containing 150 ppm MMS from 1 day before infection until 1 day after infection, and then transferred to MMS-free media. Two weeks after infection, both the treated seedlings and control seedlings were GUS assayed. On average, the frequency of treated seedlings displaying GUS activity was 18.5%, compared to 4% for control seedlings. Thus, a higher concentration of MMS (150 ppm) enhances T-DNA integration with a factor of 4.6, which is lower than was determined for 50 ppm MMS.

Example 3

Fertilizer tolerance genes as screenable and selectable markers

[0282] As alternative to the transformation method described in Example 1, which eliminates the need for an undesirable marker gene, a transformation method that relies on the use of a marker gene was developed.

[0283] The first step in developing this method was to identify a gene that not only makes it possible to select or screen for transformed plant cells but one which also confers a new and beneficial trait to resulting transgenic plants. One example of such a gene provides herbicide tolerance. A more preferred example confers tolerance to cyanamide fertilizers. To identify sources of cyanamide tolerance, a selection of soil fungi were plated on potato dextrose agar (PDA) media containing 35 mg/L cyanamide. Fungi that grew vigorously on these media include *Aspergillus* sp., *Penicillium* sp., and *Cladosporium* sp.

[0284] A putative fungal cyanamide tolerance gene was amplified from *Aspergillus* DNA with HotMaster Taq DNA Polymerase (Eppendorf). The primer pair used in these reactions was 5'-
TCTAGATGTCACAGTACGGATTGTAAG -3', and 5'-
GGTCACCTCACTGCCCATCAGGGTGCCGGCTTC -3'. The amplified fragments were both inserted into the yeast expression vector pNMT1-TOPO (Invitrogen) and the bacterial vector pGEM-T (Invitrogen). Sequence analysis of the new cyanamide tolerance gene inserted into pGEM-T (designated *CAH-H1*; see SEQ ID No.: 1) revealed less than 50% homology with both the previously identified *Myrothecium verrucaria* cyanamide hydratase (*CAH*) gene (Maier-Greiner et al., *Angew Chem Int Ed Engl*, 30: 1314-1315, 1991), and a *CAH* homolog of the highly cyanamide-sensitive species *Saccharomyces cerevisiae* (Figure 2). The PNMT1-TOPO vector carrying *CAH-H1* was introduced into *Saccharomyces pombe* by using the S.c.

EasyComp Transformation Kit (Invitrogen). Functional activity of the homolog was demonstrated by growing transformed cells on Edinburgh minimal medium (Invitrogen) containing 100 mg/L ampcilin and 50 mg/L cyanamide at 30°C. After 4 days, numerous colonies were observed on plates containing *S. pombe* cells transformed with pNMT1:CAH-H1, whereas no colonies were observed on pNMT1 control plates. The new cyanamide tolerance gene can be used as selectable marker gene for plant transformation by inserting it between a functional promoter and terminator, and introducing the resulting expression cassette into plant cells.

[0285] A second new *Cah* homolog, i.e., a "cyanamide resistance gene" that comprises the nucleotide sequence depicted in SEQ ID NO.:12, was isolated from *Aspergillus terricola* using *Cah-H1*-derived primers, 5'-ATG TGT CAG AAC GAA GTT GAA GT -3' and 5'-GGT CAC CTC ACT GCC CAT CAG GGT GCC GGC TTC-3'.

[0286] Sequence analysis of the amplified gene revealed the presence of a small intron located within the coding sequence. This intron was removed by ligating two gene fragments, amplified with the primer pairs 5'-TCT AGA TGT GTC AGA ACG AAG TTG AAG-3' and 5'-GTA TAC TCG CAT GGA GTG ATT G-3', and 5'- GTA TAC CAC TAC GGA ATG GCT ATC ACA AAG CAG CAG-3' and 5'- CTG CAG TCA CTG CCC ATC AGG GGT G-3'. The predicted protein encoded by this new cyanamide resistance gene, i.e., the protein sequence depicted in SEQ ID NO.:13, shares 58% identity with the known *Cah* gene.

[0287] To develop transformation methods that include a screening step for cyanamide tolerance, vectors were created that contain the *CAH* gene (U.S. Patent No. 6,268,547). *Agrobacterium* strains carrying such a fertilizer tolerance gene driven by the sugarcane ubiquitin-4 promoter were used to infect germinating bentgrass seedlings as described above. The infected seedlings were then planted in soil and allowed to grow for six weeks in a growth chamber (25°C with a 16-hour photoperiod). The resulting plants

were spray-treated with a 2% Dormex solution (Siemer and Associates Inc, Fresno, CA), which contains 1% hydrogen cyanamide.

[0288] About a third of the plants (84 of 250) displayed a high level of tolerance, whereas the remainder of the plants developed severe leaf necrosis. The cyanamide-tolerant plants were grown to maturity, and DNA was then extracted from flowers of these plants for PCR analysis. Using the *CAH*-specific primer pair 5'- CCA ACG GAT GGA CTG CCG TTC CAG TC - 3', and 5'- CAT GGA GTG ATT GTA GGT TTC GGG AC -3', a 180-bp DNA fragment was amplified successfully from DNA of all of cyanamide-tolerant plants, indicating that the analyzed flowers contained the *CAH* gene stably integrated into the genomes of at least some of their cells. Thus, the data demonstrate that the *CAH* gene is an effective new screenable marker gene.

[0289] The eighty-four cyanamide-tolerant flowering plants were allowed to further mature and set seed. Progeny seedlings of some of these lines were planted in soil and analyzed for the presence of the *CAH* gene by performing PCR reactions on DNA isolated from these seedlings. This experiment demonstrated that an average of 20% of progeny plants contained the *CAH* gene stably integrated into their genomes (Table 6). Interestingly, this frequency is similar to those found for tobacco and *Arabidopsis* frequencies (21% and 53%, respectively), and implies the general applicability of vortex-mediated transformation methods that do not require a selection-step.

[0290] Seed of the more recalcitrant plant species *Poa pratensis* (Kentucky bluegrass) was also successfully transformed with the *CAH*-vector. Seed of the bluegrass variety Liberator was sterilized by turbo-vortexing with 20% bleach. The sterile seed was incubated for 6 days at room temperature in the dark to allow germination. Seedlings were infected with an *Agrobacterium* strain carrying the *CAH* gene as described in Example 2. The treated seedlings were transferred to soil and grown for 3 weeks at 25°C with a 16-hour photoperiod. To screen for plants that contain the *CAH* gene in a

significant portion of plant cells, plants were then sprayed with 2% Dormex. Approximately 10% (6 of 70) of plants displayed full tolerance to this spray-treatment. These plants are being vernalized and will be permitted to flower and set seed. Progenies will be tested phenotypically and molecularly to determine the frequency of plants that contain the *CAH* gene stably integrated into their genomes. This frequency is expected to be about 5-75%.

[0291] The method described above was slightly modified to include a selection step for cyanamide tolerance. Seed of the creeping bentgrass variety L-93 was sterilized, germinated, and infected with an *Agrobacterium* strain carrying a *Cah*-vector as described in Example 1. Instead of planting the treated seedlings into soil, they were transferred to tissue culture media containing auxin 2,4-D (2 mg/L) and cyanamide (37.5 mg/L), to induce callus formation, and to select for transformation events, respectively. Surprisingly, a large percentage of seedlings (20%) developed rapidly proliferating cyanamide-tolerant callus tissue on their shoot apices, mostly around the crown region, within about 4 weeks. These calli were transferred to new MS media with a lower concentration of 2,4-D (0.01 mg/L) to induce shoot formation. Emerging shoots that arose from calli within about two weeks were transferred to MS medium lacking 2,4-D to induce root formation. After two more weeks, sufficient root mass was established, and plantlets were transferred to soil. The resulting regenerated plants displayed high levels of tolerance to spray-treatment with Dormex, and were shown by PCR to contain the *CAH* gene stably integrated into their genomes. This is the first time that whole seedlings have been used effectively as 'explant' material for the efficient transformation and subsequent proliferation and regeneration of individual plant cells. Thirty-six cyanamide tolerant plants were vernalized and allowed to set seed. Progenies derived from 2 plants were assayed by PCR to confirm the transmission of the *CAH* gene to the next generation. As shown in Table 6, the majority of tested T1 plants (5 of 6) showed positive for the transgene, implying the efficacy of this transformation method (standard

3:1 segregation ratios predict a maximum of 75% transgene-transmission to selfed progenies).

[0292] In a second experiment, creeping bentgrass seedlings were infected with *Agrobacterium* strains carrying either the *CAH* vector or a newly constructed vector containing the new cyanamide resistance gene driven by the sugarcane ubiquitin-4 promoter. Infected seedlings were transferred to callus-induction media containing either 25 or 37.5 mg/L cyanamide and treated as described above. Interestingly, the average number of calli per seedling was higher on both media for cyanamide resistance gene (244 of 450 = 0.55) than for *Cah* (160 of 450 = 0.35), and the average size was 1.6-fold larger. Thus, the functional activity of the cyanamide resistance gene as transformation marker is about 60% higher than that of *Cah*.

[0293] A further improvement was accomplished by generating a synthetic cyanamide resistance derivative gene, depicted in SEQ ID No.:14, which shares 82% identity with the original gene, and comprises codons that are optimized for expression in monocotyledonous plants. The first part of this synthetic gene was amplified by performing a PCR with the 6 primers:

[0294] 5' TCTAGAATGTGCCAAACGAGGTGGAGGTGAACGGCTGGACCTCCATGC
CAGCCAACGCCGGCGCCATTTGGCGACAAGCCATTCAAC -3'

[0295] 5' GTAGTCGAGGGTCTGGCCACCCTGGTCTCGAATGGGAACTTGATCTC
CTCGATGGAGAGGGCCTTGGCTCGTTGATGAATGGCTTGTGCCGAAG-3'

[0296] 5' GTGGTGGCCAAGACCCCTCGACTACGCCAAGGCCGTGCTCCACCCAGAG
ACCTTCAACCCTCCATGCCGTGTACCACTACGCCATGCCATACCAAG-3'

[0297] 5' GAGGTCGTGGAGGAGGCAGGTGAGGGCCCAGGTGATTGGGGAGAG
GGCGGGCGCTGCTCTGGAAATTGTTGCTTGGTATGCCATGCCGTAGTG-3'

[0298] 5' CTCACCTGCCCTCCACGACCTCGGCACCGCCGAGGAGAACCTCACC
GCCACCCGCATGCTCTCGACATCTACGGCGCATCAAGGCCCTCTCCGTG-3'

[0299] 5' GCCTCGGCGGCGGCCTCGGCTTGGTCCACGGTGGCGCCAGTCCTTGAG
CACGGAGAGGGCCTTGATGCCGCCGTAG-3' .

[0300] The product of this PCR was used for a second PCR with the primers 5'- TCT AGA ATG TGC CAA AAC GAG GTG-3' and 5'- GCC TCG GCG GCG GCC TCG GCT TGG TC -3'.

[0301] The second part of the gene was amplified with the 4 primers

[0302] 5' GCCGCCGAGGCCATCATCCGCCACGAGGACATGGCGTGGACGG
CACCATCACCTACATCGGCCAACTCATCCAACCTGCCACCACCTACGACAACAC-3'

[0303] 5' GTGTTGATTGGCGGGTCTCGTCGTGCACGAGCTGCCGAAGTCCTT
CACGTGTGGGTGGAAGCCGGTGTGTCGTAGGTGGTGGCGAGTTG-3'

[0304] 5' GACGAGACCCGCGCCCAAATCAACACCGCCTACCCACGCCCTCAAGTGGTG
CACCTTCTTCTCCGGCGTGATCCGAAGGAGGAGACATCAAGCCATGGT-3'

[0305] 5' CTGCAGTCATTGCCGTCTGGGTGCCGGCCTCGATCTCCTGTGCGAAGTC
CACGAGGTGGGTGGAGTGGCACCATGGCTTGATGGTCTCCTCCTTG-3'

[0306] The product of this PCR was used for a second PCR with the primers 5'- GCC GCC GAG GCC ATC ATC CGC CAC G -3' and 5'- CTG CAG TCA TTG GCC GTC TGG AGT G -3'. A binary vector containing the new synthetic cyanamide resistance gene of SEQ ID NO. 14 was driven by a strong promoter and can be used to generate transgenic plants that display greater levels of cyanamide tolerance than is possible with a similar construct containing either *Cah* or the new cyanamide resistance gene of SEQ ID NO. 12.

[0307] The efficacy of the new cyanamide resistance gene as superior selection marker for transformation was also tested in the dicotyledonous plant species tobacco. For this purpose, two new binary vectors were created. These vectors contain either the *Cah* gene or the cyanamide resistance gene depicted in SEQ ID NO. 12, operably linked to the potato ubiquitin-7 promoter and followed by the ubiquitin terminator. Sterile leaf disc were derived from *Nicotiana tabacum* (tobacco) variety "petite Havana SR1" plants. The leaf discs were immersed in *Agrobacterium* carrying either the *Cah* or the new cyanamide resistance gene for ten minutes

and then transferred to sterile filter paper for one minute. Infected discs were transferred on to Murashige & Shoog (MS) medium modified for tobacco tissue culture (product number M401, PhytoTechnology Laboratories, Shawnee Mission, KS) for one day. Following co-culture, leaf discs were transferred on to tobacco modified MS medium containing 300 mg/L timentin and 6.25 mg/L cyanamide. Cultures were placed in a growth chamber at 24 °C and a 16 hour photoperiod. Results indicate a 55% increase in shoot regeneration from leaf disc transformed with the cyanamide resistance gene compared to leaf discs transformed with the *Cah* gene.

[0308] The very high transformation efficiencies that can be obtained by using whole seedlings as explant material for vortex-mediated transformation make this a preferred method for applications that require high-throughput transformation procedures such as functional genomics. This method is also desirable for, for example, "proof-of-concept" experiments, and for projects related to the overexpression of pharmaceutical and nutraceutical proteins and peptides in plants.

[0309] Generating a codon-optimized synthetic gene can further enhance the functional activity of cyanamide resistance protein in dicotyledonous plants. This alternative cyanamide resistance gene derivative is optimized, therefore, for expression in dicotyledonous plants, particularly potato, and is depicted in SEQ ID NO. 15.

[0310] Four primers used to generate the first part of such a gene are:

[0311] 5' ATGTGTCAGAATGAAGTTGAAGTTAATGGATGGACTTCTATG
CCAGCTAACATGCTGGAGCTATCTTGGAGATAAGCCATTATTAATGAACCAAAG-3'

[0312] 5' CAAGAGTCCTAGCAACAACTGGATCATCAAATGGAAACTTAATTCTT
CAATAGAAAGAGCCTTGTTGTTCAATTAATAATGGCTTATCTC-3'

[0313] 5' GATCCAGTTGTTGCTAAGACTCTGATTATGCTAAGGCTGTTCTTCAT
CCAGAAACTTTAACATTCTATGAGAGTTATCATTATGGAATG-3'

[0314] 5' GGGCCCAAGTAATTGGAGAAAGAGCAGCAGCTTGTCTGAAATTGTTG
CTTAGTAATGCCATTCCATAATGATAAACTCTCATAG-3' .

[0315] This first gene part was re-amplified with the primers 5'-GGA
TCC ATG TGT CAG AAT GAA GTT GAA G-3' and 5'-GGG CCC AAG TAA
TTG GAG AAA GAG C-3'.

[0316] Six primers for the second part are:

[0317]

5' GGGCCCTTACTTGTCTTCATGATCTTGAACGTGCTGAAGAGAATCTTACTGCTACTAGAATGTC
TTTGATATTTATGGAGGAATTAAGGCTC-3'

[0318]

5' CATGTCTAATAATAGCTTCAGCAGCAGCTTCAGCTTGATCAACAGTAGCTCCGAAATCCTTAAGAAC
AGAAAAGAGCCTTAATTCCCTCCATAAAATATC-3'

[0319]

5' GCTGCTGAAGCTATTATTAGACATGAAGATATGGAGTTGATGAACTATTACTTATATTGGACAAC
TTATTCAACTTGCTACTACTTATGATAATAC-3'

[0320]

5' GCAGTATTAATTGAGCCCTAGTTCATCATGAACAAAGTTACCAAAATCCTAACATGTGGATGAA
ATCCAGTATTATCATAAGTAGTAGCAAGTTG-3'

[0321]

5' GAAACTAGGGCTCAAATTAAATACTGCTTATCCAAGACTTAAGTGGTGTACATTCTTCTGGAGTTA
TTAGAAAGGAAGAAACTATTAGGCATGG-3'

[0322] 5' GAGCTCTTATTGTCCATCTGGAGTTCCAGCTCAATTCTTATCAAAATC
ACAAGATGAGTAGAATGACACCAGGCTTAATAGTTCTTCTTTC-3' .

[0323] The PCR product was re-amplified with the primers 5'-GGG
CCC TTA CTT GTC TTC TTC ATG-3' and 5'-GAG CTC TTA TTG TCC ATC
TGG AGT-3'. The sequence of the ligated DNA fragments representing the
codon-optimized gene is shown in SEQ ID NO. 15.

[0324] A transformation process that includes a selection step for
cyanamide tolerance was also applied to Kentucky bluegrass (*Poa pratensis*).

Seed of the bluegrass variety "Liberator" was sterilized, germinated, and infected with an *Agrobacterium* strain carrying a *Cah*-vector as described in Example 1. Instead of planting the treated seedlings into soil, they were transferred to tissue culture media containing auxin 2,4-D (2 mg/L) and cyanamide (37.5 mg/L), to induce callus formation, and to select for transformation events, respectively. Again, a large percentage of seedlings (376 of 2500 = 15%) developed rapidly proliferating cyanamide-tolerant callus tissue on their shoot apices, mostly around the crown region, within about 4 weeks. These calli were transferred to new MS media with a lower concentration of 2,4-D (0.01 mg/L) to induce shoot formation. Emerging shoots that arose from about 7% of calli within the following two months were transferred to MS medium lacking 2,4-D to induce root formation and generate whole plants.

[0325] The above-described transformation method is also applicable to other plant species such as maize and alfalfa. Sterilized seeds are germinated for 2 and 3 days, respectively, and infected with an *Agrobacterium* strain carrying a *Cah*-vector as described in Example 1. The infected maize seedlings are then transferred to a callus-induction medium, such as MS containing 2,4-D (1 mg/L), BA (2 mg/L), proline (4 g/L), and cyanamide (37.5 mg/L), and allowed to develop cyanamide-tolerant calli with an efficiency of up to 80%. These calli can then be transferred to an appropriate regeneration media, allowed to root, and transferred to soil.

[0326] In a similar way, infected alfalfa seedlings are transferred to a callus-induction medium, such as Schenk and Hildebrandt (SH) containing 2,4-D (2 mg/L), kinetin (2 mg/L) and about 6.5 mg/L cyanamide. The treated seedlings developed calli with an efficiency of up to 80%. These calli were transferred to regeneration media, allowed to root, and transferred to soil.

Example 4

New binary vectors for transformation of plants

[0327] Current methods to express a foreign gene in crop plants result in the introduction of various nucleic acids that are derived from non-food sources. The introduction of such DNA in the food supply is undesirable and should be limited or avoided. The current invention provides tools and methods to (1) replace the *Agrobacterium*-derived T-DNA with a DNA fragment derived from a food source, (2) prevent transformation events that contain bacterial vector backbone sequences from developing into whole plants, (3) replace the frequently used nopaline synthase (nos) terminator derived from *Agrobacterium* with a terminator derived from a food source, and (4) replace frequently used virus promoters with promoters derived from food sources.

1. New transfer DNA

[0328] The *Agrobacterium*-derived T-DNA is delineated by a 25-bp left-border (LB) and right-border (RB) repeat, which function as specific recognition sites for virD2-catalyzed nicking reaction (Schilperoort et al., U.S. Patent 4940838, 1990). The single stranded DNA released by these nicking reactions is transferred to plant cell nuclei where it often successfully integrates into the plant genome. Advanced BLAST searches of public databases including those maintained by The National Center For Biotechnology Information and SANGER failed to identify any border sequences in plants. It was therefore necessary to consider plant DNA sequences that are similar but not identical to T-DNA borders, designated here as "border-like". The challenge in trying to replace T-DNA borders with border-like sequences is that border sequences are highly conserved (see Table 1). A large part of these sequences is also highly conserved in the nick regions of other bacterial DNA transfer systems such as that of IncP, PC194, and fX174, indicating that these sequences are essential for conjugative-like

DNA transfer (Waters et al., *Proc Natl Acad Sci* 88: 1456-60, 1991).

Because there are no reliable data on border sequence requirements, the entire border seems therefore important in the nicking process. A single study that attempted to address this issue by testing the efficacy of border mutants in supporting DNA transfer is unreliable because negative controls did not appear to function appropriately (van Haaren et al., *Plant Mol Biol* 13: 523-531, 1989). Furthermore, none of the results of this study were confirmed molecularly. Despite these concerns, two possibly effective border mutants are shown in Table 1 as well.

[0329] Based on the homology among border sequences, a T-DNA border motif was identified (Table 1). Although this motif comprises 13,824 variants, many of which may not function –or may be inadequate- in transferring DNA, it represents the broadest possible definition of what a T-DNA border sequence is or may be. This border motif was then used to search publicly available DNA databases for homologs using the “Motif Alignment and Search Tool” (Bailey and Gribskov, *Bioinformatics* 14: 48-54, 1998) and “advanced BLASTN” (“penalty for nucleotide mismatch” = -1; “expect” = 105; Altschul et al., *Nucleic Acids Res* 25: 3389-3402, 1997). Again, these searches did not identify any identical matches in organisms other than *Agrobacterium*.

[0330] To try and increase the chance of isolating a plant DNA fragment containing border-like sequences that correspond to the border motif, DNA was isolated from 100 genetically diverse potato accessions (the so-called “core collection,” provided by the US Potato Genebank, WI). This DNA was pooled and used as template for polymerase chain reactions using a variety of oligonucleotides designed to anneal to borders or border-like sequences. Amplified fragments were sequence analyzed, and the sequence was then confirmed using inverse PCR with nested primers. One of the potato DNA fragments that was of particular interest contains a novel sequence without any major open reading frames that is delineated by border-

like sequences (Table 1). One of the border-like sequences of this fragment contains 5 mismatches with the closest T-DNA border homolog; the other border-like sequence contains 3 mismatches with the closest homolog. Although both sequences contain one mismatch with the border motif, they were tested for their ability to support DNA transfer. For that purpose, the fragment was first reduced in size to 0.4-kilo basepairs by carrying out an internal deletion (SEQ ID NO.: 2). The resulting fragment was designated "P-DNA" (plant DNA) to distinguish it from the *Agrobacterium*-derived T-DNA.

[0331] To test the efficacy of P-DNA transfer from *Agrobacterium* to plant cells, an expression cassette for the neomycin phosphotransferase (NPTII) gene was inserted within the P-DNA sequence, located on a T-DNA-free plasmid that can be maintained in both *E. coli* and *A. tumefaciens*. An *Agrobacterium* strain carrying the resulting vector was used to infect stem explants of 4-week-old *in vitro* grown plantlets of the potato variety Russet Ranger. The infected stems were incubated for 2 days on co-culture medium (1/10 MS salts, 3% sucrose, pH 5.7) containing 6 g/L agar at 22°C in a Percival growth chamber (16 hrs light) and subsequently transferred to callus induction medium (CIM, MS medium supplemented with 3% sucrose 3, 2.5 mg/L of zeatin riboside, 0.1 mg/L of naphthalene acetic acid, and 6g/L of agar) containing timentine (150 mg/L) and kanamycin (100 mg/L). After 1 month of culture on CIM, explants were transferred to shoot induction medium (SIM, MS medium supplemented with 3% sucrose, 2.5 mg/L of zeatin riboside, 0.3 mg/L of giberelic acid GA3, and 6g/L of agar) containing timentine and kanamycin (150 and 100 mg/L respectively). After 3-4 weeks, the number of explants developing transgenic calli and/or shoots was counted. More calli were observed on potato stem explants infected with an *Agrobacterium* strain containing the P-DNA vector (0.59 calli/explant) than on explants infected with the control T-DNA vector pBI121 (Genbank accession number AF85783) (0.31 calli/explant).

[0332] Turf seedlings were also infected with a modified P-DNA vector comprising a ubiquitin-4 promoter driving GUS expression. GUS assays on the transformed plants showed that transformation efficiency were similar to those with control T-DNA vectors.

2. Cytokinin genes as backbone-integration markers

[0333] To make it possible to select against the frequent occurrence of backbone integration events, an expression cassette comprising the *Agrobacterium isopentenyl transferase (IPT)* gene driven by the Ubi3 promoter and followed by the Ubi3 terminator (SEQ ID NO.: 3) was inserted as 2.6 kbp SacII fragment into the backbone of the P-DNA vector described above.

[0334] Transformed shoots, generated by infecting potato leaf explants as described above, could be grouped into two different classes. The first class of shoots (55 of 193) was phenotypically indistinguishable from control shoots transformed with LBA::pBI121. The second class of shoots (138 of 193) displayed an IPT phenotype. Shoots of the latter class were stunted in growth, contained only very small leaves, displayed a light-green to yellow color, and were unable to root upon transfer to hormone-free media. To confirm that shoots with an IPT phenotype contained the IPT gene stably integrated in their genomes, all shoots were transferred to Magenta boxes containing MS medium supplemented with 3% sucrose and timentine 150 mg/L, allowed to grow for 3 to 4 additional weeks, and used to isolate DNA. This plant DNA served as template in PCR reactions with an oligonucleotide pair designed to anneal to the IPT gene: 5'- GTC CAA CTT GCA CAG GAA AGA C-3', and 5'- CAT GGA TGA AAT ACT CCT GAG C-3'. This PCR experiment confirmed a strict correlation between IPT phenotype and presence of the *IPT* gene. A second PCR experiment was carried out to test whether IPT-free plants did not contain any other backbone sequences. Because the IPT expression cassette is positioned close to the left border-like sequences, the oligonucleotide pair for this experiment was designed to

anneal to backbone sequences close to the right border-like sequence: 5'-CAC GCT AAG TGC CGG CCG TCC GAG-3', and 5'-TCC TAA TCG ACG GCG CAC CGG CTG-3'. Data from this experiment confirm that plants that are positive for the *IPT* gene are also positive for this other part of the backbone.

3. New terminators

[0335] Instead of the frequently used bacterial terminator of the nopaline synthase gene, a new sequence derived from a food source was used to terminate transcription of a selectable marker gene. This terminator is the yeast alcohol dehydrogenase-1 (ADH1) terminator (Genbank accession number V01292, SEQ ID NO. 4). Surprisingly, this specific yeast terminator was shown to function effectively in plant cells by Agro-infecting potato stem explants with different binary vectors that carry an intron-containing *GUS* gene operably linked to the Ubi7 promoter and followed by either that terminator or the yeast CYC1 terminator. Five days after infection, high levels of transient GUS expression were monitored with the ADH1 terminator, whereas almost no GUS expression was detected with the CYC1 terminator. To terminate transcription of a desired polynucleotide in dicotyledonous plant species, the potato Ubiquitin-3 terminator was used (SEQ ID NO.:5). For transcriptional termination in monocotyledonous plant species, a new terminator was amplified from DNA of the rice variety "Lemont", where it is associated with the actin-1 gene, with the primer set: 5'-GGATCCTCGTCATTACTTTATCTTAATGAGC -3' and 5'-GAATTCACATTATAAGCTTATATTACCAAGG -3' (SEQ ID NO.:6). Functional activity of this rice terminator was demonstrated by operably linking it to a promoter-GUS fusion. Five days after infecting bentgrass seedlings with an *Agrobacterium* strain containing the resulting expression cassette between borders of a binary vector, transient GUS expression levels were equally high as with a control experiment based on a similar vector carrying the frequently used terminator of the bacterial nopaline synthase gene.

4. New promoters

[0336] Instead of viral promoters such as the 35S promoter of cauliflower mosaic virus, new plant promoters were developed and used to express genes in transgenic plants. For some important dicotyledonous plants including potato and cotton, a new promoter was isolated from the potato genome. This new promoter represents a small part (492-bp) of the previously described 1220-bp and 1788-bp promoters of the potato Ubiquitin-7 gene (Garbarino et al., U.S. Patent 6,448,391 B1, 2002). This conveniently-sized fragment (SEQ ID NO.: 7) was tested for its efficacy to promote high-level expression of transgenes by Agro-infecting tobacco explants with a binary vector carrying the fragment operably linked to the *NPTII* gene, and placing the infected explants on MS media containing 100 mg/L kanamycin. Within two weeks, a large number of calli developed on these explants, whereas explants infected with a control strain did not contain any calli. Apart from tobacco, the small new promoter was also shown to be active in potato and cotton. An alternative promoter that can be used to drive high-level expression represents 1,026-bp of the Ubi7 promoter (SEQ ID NO.: 8).

[0337] For monocotyledonous plants, a promoter was developed that resembles the sugarcane ubiquitin-4 promoter. The sequence of this small promoter, designated UbiN, is shown in SEQ ID NO.:9; its homology with the corresponding part of the original Ubiquitin-4 promoter is shown in Figure 3. The functional activity of UbiN was assessed by first inserting it between a small HindIII – Sall 0.2-kbp DNA fragment (SEQ ID NO.: 10) isolated from a modified maize matrix attachment region using the primer set: 5'- AAG CTT AAT AGC TTC ACC TAT ATA ATA -3', and 5'- GTC GAC GGC GTT TAA CAG GCT -3', and a modified EcoRI – BamHI 1.4-kbp fragment containing an intron associated with a sugarcane ubiquitin gene, using the primer set 5'- GAA TTC CCT TCG TCG GAG AAA TTC ATC GAA G -3', and 5'- GGA TCC CTG CAA GCA TTG AGG ACC AG -3' (SEQ ID NO.: 11). The fused DNA fragments were then operably linked to the CAH gene followed by a

terminator, and a binary vector containing this expression cassette was used to Agro-infect bentgrass seedlings as described in Example 1. Vigorously growing calli demonstrated that the sugarcane-derived promoter is effective in promoting transgene expression.

5. New vectors

[0338] As shown in Figure 1, a vector of the present invention may comprise, in 5'- to 3'- orientation, (i) a cytokinin gene (the backbone integration marker) operably linked to elements that can express it, (ii) a first border(-like) P-DNA sequence, (iii) a desired polynucleotide that is operably linked to a promoter and terminator, (iv) an optional selectable marker that is operably linked to a promoter and a terminator, which is associated with a gene that is not naturally expressed in plants, and (v) a second border(-like) P-DNA sequence. A vector also may comprise another desired polynucleotide operably linked to a promoter and terminator, preferably derived from food sources, and inserted within the T-DNA or P-DNA sequence.

Example 4

Further enhancement of marker-free transformation efficiencies

[0339] Example 1 describes new plant transformation methods that are based on the turbo-vortexing of seedlings in solutions containing *Agrobacterium*. These methods results in very high transformation frequencies, thus making it unnecessary to use selectable marker genes. Control experiments that omit tubo-vortexing result in very few, if any, transformation events. For instance, Figure 4 shows the difference in alfalfa transformation frequencies with and without turbo-vortexing.

[0340] Turbo-vortex mediated transformation frequencies can be further enhanced by subjecting seedlings to purine synthesis inhibitors such as mizoribine (about 10-50 µg/mL), azaserine (about 20-100 µg/mL), and acivicin (about 20-100 µg/mL) for about 16 hours prior to infection. The

inhibitors are easily applied to germination media in concentrations that do not negatively affect plant growth.

Tables**Table 2. *Arabidopsis* transformation in T0**

<i>Experiment</i>	<i>Transgene</i>	<i>Treatment</i>	<i>GUS-positive seedlings</i>
85-6	<i>GUS</i>	5-min. vortex	15% (11 of 74)
90-1	<i>GUS</i>	5-min. vortex	21% (16 of 75)
95-1	<i>GUS</i>	5-min. vortex	17% (30 of 181)
91-1	<i>GUS</i>	5-min. vortex	5% (3 of 62)
92-1	<i>GUS</i>	5-min. vortex	18% (15 of 183)
90-3	<i>GUS</i>	5-min. vortex	16% (14 of 87)
<i>AVERAGE</i>	<i>GUS</i>	5-min. vortex	13% (89 of 662)
85-5	<i>GUS</i>	30-min. vortex	15% (11 of 74)
90-2	<i>GUS</i>	30-min. vortex	7% (5 of 69)
91-2	<i>GUS</i>	30-min. vortex	9% (4 of 47)
91-4	<i>GUS</i>	30-min. vortex	3% (2 of 80)
90-4	<i>GUS</i>	30-min. vortex	1% (1 of 72)
92-4	<i>GUS</i>	30-min. vortex	11% (14/123)
63-2	<i>GUS</i>	30-min. vortex	32% (27/84)
63-3	<i>GUS</i>	30-min. vortex	24% (15/63)
<i>AVERAGE</i>	<i>GUS</i>	30-min. vortex	13% (79 of 612)

Table 3. Transgenic *Arabidopsis* plants in selfed progeny

<i>Experiment</i>	<i>Transgene</i>	<i>GUS-positive seedlings</i>
63-2-67	<i>GUS</i>	37% (43 of 117)
63-6-16	<i>GUS</i>	51% (55 of 108)
63-3-57	<i>GUS</i>	71% (36 of 51)
63-3-60	<i>GUS</i>	64% (54 of 85)
78-8-34	<i>GUS</i>	56% (53 of 94)
63-2-22	<i>GUS</i>	48% (73 of 153)
63-3-12	<i>GUS</i>	48% (70 of 147)
69-2-60	<i>GUS</i>	78% (53 of 68)
<i>AVERAGE</i>	<i>GUS</i>	53% (437 of 823)

Table 4. Tobacco transformation in T0

<i>Experiment</i>	<i>Transgene</i>	<i>Treatment</i>	<i>GUS-positive seedlings</i>
94-1	<i>GUS</i>	5-min. vortex	4% (4 of 94)
91-5	<i>GUS</i>	5-min. vortex	0% (0 of 74)
94-2	<i>GUS</i>	5-min. vortex	7% (7 of 100)
91-6	<i>GUS</i>	5-min. vortex	1% (1 of 75)
75-1	<i>GUS</i>	5-min. vortex	8% (15 of 194)
78-5	<i>GUS</i>	5-min. vortex	19% (17 of 91)
<i>AVERAGE</i>	<i>GUS</i>	5-min. vortex	7% (44 of 628)
85-2	<i>GUS</i>	30-min. vortex	0% (0 of 23)
92-6	<i>GUS</i>	30-min. vortex	0% (0 of 127)
73-2	<i>GUS</i>	30-min. vortex	10% (16 of 155)
73-1	<i>GUS</i>	30-min. vortex	5% (7 of 135)
70-3	<i>GUS</i>	30-min. vortex	8% (4 of 51)
68-3	<i>GUS</i>	30-min. vortex	0% (0 of 49)
60-1	<i>GUS</i>	30-min. vortex	2% (2 of 83)
68-1	<i>GUS</i>	30-min. vortex	8% (5 of 61)
80-1	<i>GUS</i>	30-min. vortex	2% (2 of 97)
80-3	<i>GUS</i>	30-min. vortex	7% (4 of 54)
85-1	<i>GUS</i>	30-min. vortex	4% (1 of 27)
<i>AVERAGE</i>	<i>GUS</i>	30-min. vortex	5% (41 of 862)

Table 5. Transgenic tobacco plants in selfed progeny

<i>Experiment</i>	<i>Transgene</i>	<i>GUS-positive seedlings</i>
62-3-11	<i>GUS</i>	12% (10 of 85)
70-3-18	<i>GUS</i>	15% (16 of 110)
70-3-23	<i>GUS</i>	27% (74 of 275)
70-4-49	<i>GUS</i>	22% (20 of 91)
<i>AVERAGE</i>	<i>GUS</i>	21% (120 of 561)

Table 6. Transgenic creeping bentgrass in selfed progeny

<i>Experiment</i>	<i>Transgene</i>	<i>Treatment in T0</i>	<i>CAH-positive seedlings</i>
5G-12	<i>CAH</i>	Dormex screen	0 of 4
5G-18	<i>CAH</i>	Dormex screen	1 of 1
5G-23	<i>CAH</i>	Dormex screen	4 of 21
5G-24	<i>CAH</i>	Dormex screen	2 of 12
5G-29	<i>CAH</i>	Dormex screen	0 of 7
5G-31	<i>CAH</i>	Dormex screen	1 of 8
3B-7	<i>CAH</i>	Dormex screen	1 of 3
3B-14	<i>CAH</i>	Dormex screen	0 of 1
AVERAGE	<i>CAH</i>	Dormex screen	16% (9 of 57)
5J-18	<i>CAH</i>	Cyanamide selection	1 of 1
5J-23	<i>CAH</i>	Cyanamide selection	4 of 5
3F-11	<i>CAH</i>	Cyanamide selection	1 of 2
AVERAGE	<i>CAH</i>	Cyanamide selection	~75% (6 of 8)

SEQ ID NOs.

SEQ ID NO.:1 Cyanamide tolerance gene from *Aspergillus* sp.

SEQ ID NO.:2 Potato P-DNA. The bold underlined portions represent the left (5') and right (3') border-like sequences of the P-DNA respectively.

SEQ ID NO.:3 Expression cassette for the cytokinin *IPT* gene

SEQ ID NO.:4 Terminator associated with the yeast *ADH1* gene

SEQ ID NO.:5 Terminator associated with the potato Ubiquitin-3 gene

SEQ ID NO.:6 Terminator associated with the rice actin-1 gene

SEQ ID NO.:7 Short 0.5-kbp promoter associated with the potato Ubiquitin-7 gene

SEQ ID NO.:8 Short 1.0-kbp promoter associated with the potato Ubiquitin-7 gene

SEQ ID NO.:9 Plant-like promoter

SEQ ID NO.:10 Part of a maize matrix-associated region

SEQ ID NO.:11 Intron associated with the sugarcane Ubiquitin-4 gene

SEQ ID NO.:12 New cyanamide resistance gene nucleotide sequence

SEQ ID NO.:13 New cyanamide resistance protein sequence

SEQ ID NO.:14 Nucleotide sequence of a new cyanamide resistance gene that is codon optimized for expression in a monocotyledonous plant

SEQ ID NO.:15 Nucleotide sequence of a new cyanamide resistance gene that is codon optimized for expression in a dicotyledonous plant

SEQ ID No. 1

ATGTGTCAGAACGAAGTGAAGTCAATGGCTGGACCAGCATGCCTGCTGATGCTGGGCCATCTTGATGGTGGACCCCTCATCACGAACTGGCTGCTTCCCAGTCAAGAGAGATCAAGTTCCAGTCGATGACCCCATTGTTGAGAAAACCATGAGATATGCAAAGGCTGCTTCCCAGTCAACACTCTATGAGA GTTTACTATTACGGTATGCAGGACTGCAGCTCCCATGGTGTCTTAATCAATCGCTCACAGGCTCTAGGA ATGGCTATCACCAGCAATTCCGAAAGCAAGGCAAGCTGCCCTTAGGCCAGTACCTGGGCCATGACCTGGCTGACATCGTACTTCCGACCACACCTCGCTGCAACTCGCATGTCCTTGATATCTAC GGTGGTATCAAGGCTCTGGAGGTTCTAAGGGTTGGCGTACCTCCGATCAGGCCAGCTCATCCAG GAGGCCATCATCCGACACCAGGATCTGGAGTTCACTGGGACGATCACGTATATCGGCCAGCTCATCCAG CTGGCCACCATCTACGATAACGTGGGCTCACCTTACGTCAAAGACTTGGCAGTTGATCCATGAT ACAACTCGCTCCACGGTCCACGAGGCCACCCGCCGGGAATGGGCCACGTTCTCTGGCGTCATC CAGAAGGAGCAAGCAATCAAGCCCTGGTGTACACAAAAAGATGGTGAATGTTGAGGAAAGGAAGC CGGCACCCGTATGGGAGTGA

SEQ ID No. 2

GTTTACATTACCATATATCCTGTCAGAGGTATAGAGGCATGACTGGCATGATCACTAAATTGATGCCA CAGAGGAGACTTATAACCTACAGGGCACGTAGTCTAGGACTTGAAAGTGACTGACCGTAGTCCAAC TCGGTATAAAGCCTACTCCAACTAAATATATGAAATTATAGCATAACTGCAGATGAGCTGATTCTAG AGTAGGTACCGAGCTCGAATTCTTACTCCTCCACAAGCCGTAAC TGAGCAGCTTCTATTTCATCA ACCTTCGGACCTGACGATCAAGAATCTCAATAGGTAGTTCTCATAGTGAAGACTATCCTCATAGCTA CACTTCTAAAGGTACGATAGATTGGATCAACCACACACTTCGTTTACATCGTATATACTCCTGC CA

SEQ ID No. 3

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SEQ ID No. 4

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SEQ ID No. 5

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SEQ ID No. 6

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SEQ ID No. 7

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SEQ ID No. 8

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SEQ ID No. 9

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SEQ ID No. 10

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SEQ ID No. 11

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SEQ ID No. 12

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SEQ ID No. 13

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VYHYGMAITKQQFPEQAAALSPITWALTCLLHD LGTAEE NL TATRMS FDI YGGIKAL SVLKDFG ATVDQ
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SEQ ID No. 14

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SEQ ID No. 15

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WHAT IS CLAIMED IS:

1. A method for producing a transgenic plant, comprising (a) agitating a solution, which comprises (1) a germinating plant seedling, or explant thereof, and (2) at least one Agrobacterium strain that comprises a vector, which comprises a desired polynucleotide; (b) cultivating the seedling to produce a plant; and (c) screening the plant to determine if the desired polynucleotide is integrated into the genome of at least one cell of the plant to produce a stably transformed plant, wherein the step of agitating the solution does not comprise sonication, and wherein the germinating plant seedling is exposed to an agent that enhances transformation efficiency before, during, or after the step of agitating the solution.
2. The method of claim 1, wherein the agent that enhances transformation efficiency is at least one of a purine inhibitor, a pyrimidine inhibitor, or a purine- and a pyrimidine- inhibitor.
3. The method of claim 1, wherein the agent is selected from the group consisting of mizoribine, azathioprine, mycophenolic acid, mycophenolate mofetil, 5-fluorouracil, Brequinar sodium, leflunomide, azaserine, acivicin, methotrexate, methotrexate polyglutamate derivatives, and cyclophosphamide.
4. The method of claim 2, wherein the agent is a purine inhibitor and a pyrimidine inhibitor.
5. The method of claim 4, wherein the agent is azaserine or acivicin.
6. The method of claim 1, wherein the agent induces chromosome breakage.

7. The method of claim 6, wherein the agent is methyl methane sulfonate.

8. The method of claim 1, wherein the vector comprises (a) a T-DNA or a P-DNA, which comprises (i) the desired polynucleotide, and (ii) a selectable marker gene operably linked to a terminator that is not naturally expressed in plants; and (b) a backbone integration marker gene, wherein the desired polynucleotide and the selectable marker gene are positioned between the border sequences of the T-DNA or between the border-like sequences of the P-DNA, and wherein the backbone integration marker gene is not positioned within the T-DNA or within the P-DNA.

9. The method according to claim 8, further comprising (i) producing a callus from the cultivated seedling; and (ii) inducing shoot and root formation from the callus, prior to transferring to soil to produce the plant.

10. The method of claim 9, wherein the step of producing the callus from the transformed seedling comprises (i) transferring the seedling that had been subjected to agitation to tissue culture media, which contains auxin and cyanamide; (ii) selecting a fertilizer-resistant callus; (iii) inducing shoot and root formation from the callus; and (iv) transferring a callus with shoots and roots to soil and exposing the callus to conditions that promote growth of a transgenic plant from the callus.

11. The method of claim 10, wherein expression of the selectable marker gene confers fertilizer resistance or cyanamide resistance to the transgenic plant and to progeny of the transgenic plant

12. The method of claim 11, wherein the selectable marker gene is a cyanamide resistance gene.

13. The method of claim 12, wherein the cyanamide resistance gene comprises the nucleotide sequence depicted in any one of SEQ ID NO. 12 or a variant thereof, SEQ ID NO. 14 or a variant thereof, or SEQ ID NO. 15 or a variant thereof, and wherein the gene encodes a protein that confers cyanamide resistance.

14. The method of claim 13, wherein the protein that confers cyanamide resistance comprises the sequence of SEQ ID NO. 13 or a variant thereof, wherein the variant protein is functionally active.

15. The method of claim 13, wherein the germinating plant seedling or explant thereof is a monocotyledonous plant and the cyanamide resistance gene (i) comprises the sequence of SEQ ID NO. 14, or a variant thereof, and (ii) encodes a functional cyanamide resistance protein.

16. The method of claim 13, wherein the plant seedling or explant thereof is a dicotyledonous plant and the cyanamide resistance gene (i) comprises the sequence of SEQ ID NO. 15, or a variant thereof, and (ii) encodes a functional cyanamide resistance protein.

17. The method of claim 1, wherein the germinating plant seedling is from a monocotyledonous plant.

18. The method of claim 17, wherein the monocotyledonous plant is selected from the group consisting of bentgrass, bluegrass, turfgrass, wheat, maize, rice, oat, barley, orchid, iris, lily, onion, sugarcane, and sorghum.

19. The method of claim 9, wherein the turfgrass is selected from the group consisting of *Agrostis spp.*, *Poa pratensis*, *Lolium spp.*, *Festuca arundinacea*, *Festuca rubra commutata*, *Cynodon dactylon*, *Pennisetum*

clandestinum, *Stenotaphrum secundatum*, *Zoysia japonica*, and *Dichondra micrantha*.

20. The method of claim 1, wherein the germinating plant seedling is from a dicotyledonous plant.

21. The method of claim 20, wherein the dicotyledonous plant is selected from the group consisting of cotton, tobacco, *Arabidopsis*, tomato, potato, sugar beet, broccoli, cassava, sweet potato, pepper, poinsettia, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, geranium, and cactus.

22. The method of claim 1, wherein expression of the desired polynucleotide in the stably transformed plant confers a trait to the plant selected from the group consisting of increased drought tolerance, reduced height, enhanced cold and frost tolerance, improved vigor, enhanced color, enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced sweetness, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity, and production of novel proteins or peptides.

23. The method of claim 1, wherein the desired polynucleotide expresses a peptide or protein that is an antifungal, a nutritional peptide or protein, a transcription factor, a receptor that binds to pathogen-derived ligands, a hemoglobin, an oxidase, an enzyme of the lignin biosynthesis pathway, an enzyme of industrial value, or an antigen.

24. The method of claim 8, wherein the backbone integration marker is a cytokinin gene.

25. The method of claim 24, wherein the cytokinin gene is *IPT*, and the plant is a dicotyledonous plant.

26. The method of claim 8, wherein the backbone integration marker is *PGA22*, *TZS*, *HOC1*, *CKI1*, and *ESR1*.

27. The method of claim 1, wherein the step of agitating the solution is accomplished by vortexing.

28. The method of claim 27, wherein the solution is vortexed from about 60 seconds to several hours.

29. The method of claim 28, wherein the solution is vortexed for about 5 minutes to about 30 minutes.

30. The method of claim 1, wherein the step of cultivating the seedling to produce a transgenic plant comprises transferring the *Agrobacterium*-transformed seedling to soil, and exposing the transformed seedling to conditions that promote growth.

31. The method of claim 1, wherein the step of cultivating the seedling to produce a transgenic plant comprises cultivating the *Agrobacterium*-transformed seedling in or on tissue culture medium prior to transferring the transformed seedling to soil, and exposing the transformed seedling to conditions that promote growth.

32. The method of claim 1, wherein the transformed plant seedling is grown to maturity, crossed to a non-transformed plant and the desired polynucleotide transmitted to at least one progeny plant.

33. The method of claim 1, wherein the transformed plant seedling is grown to maturity, selfed, and the desired polynucleotide transmitted to progeny.

34. A method for producing a transgenic plant, comprising
(a) agitating a solution that comprises (1) a germinating plant seedling and (2) at least one *Agrobacterium* strain that comprises a vector, which comprises (i) a desired polynucleotide and (ii) a cyanamide resistance gene; (b) (i) producing a callus from the transformed seedling and (ii) inducing shoot and root formation from the callus to produce plantlets; (c) growing the plantlets into plants; and (d) screening the plants to determine if the desired polynucleotide is incorporated into the genome of at least one cell of the plant to produce a stably transformed transgenic plant, and wherein the step of agitating the solution does not comprise sonication.

35. The method of claim 34, wherein the desired polynucleotide and the cyanamide resistance gene, which is operably linked to a terminator that is not naturally expressed in plants, are positioned between border or border-like sequences of a T-DNA or a P-DNA located in the vector.

36. The method of claim 35, wherein the cyanamide resistance gene comprises the nucleotide sequence of any one of SEQ ID NOs. 12 or a variant thereof, SEQ ID NO. 14 or a variant thereof, or SEQ ID NO. 15 or a variant thereof, and wherein the cyanamide resistance gene encodes a protein that comprises the amino acid sequence of SEQ ID NO. 13.

37. The method of claim 36, wherein the vector further comprises a backbone integration marker gene, which is not positioned between the border or border-like sequences of the T-DNA or the P-DNA.

38. The method of claim 34, further comprising exposing the germinating plant seedling to an agent that enhances transformation efficiency.

39. The method of claim 38, wherein the agent that enhances transformation efficiency is at least one of a purine inhibitor, a pyrimidine inhibitor, or a purine- and a pyrimidine- inhibitor.

40. The method of claim 39, wherein the agent is selected from the group consisting of mizoribine, azathioprine, mycophenolic acid, mycophenolate mofetil, 5-fluorouracil, Brequinar sodium, Leflunomide, azaserine, acivicin, methotrexate, methotrexate polyglutamate derivatives, and cyclophosphamide.

41. The method of claim 34, wherein the agent induces chromosomal breakage.

42. The method of claim 41, wherein the agent is methyl methane sulfonate.

43. The method of claim 34, wherein the step of agitating the solution is accomplished by vortexing.

44. The method of claim 43, wherein the solution is vortexed from about 60 seconds to several hours.

45. The method of claim 44, wherein the solution is vortexed for about 5 minutes to about 30 minutes.

46. An isolated nucleic acid comprising the sequence of SEQ ID NO. 12, or variant thereof, wherein the nucleic acid encodes a functional cyanamide resistance protein.

47. The isolated nucleic acid of claim 46, wherein the cyanamide resistance protein comprises the amino acid sequence of SEQ ID NO. 13 or variant thereof.

48. An isolated nucleic acid comprising the sequence of SEQ ID NO. 14, or variant thereof, wherein the nucleic acid encodes a functional cyanamide resistance protein.

49. The isolated nucleic acid of claim 48, wherein the cyanamide resistance protein comprises the amino acid sequence of SEQ ID NO. 13 or variant thereof.

50. An isolated nucleic acid comprising the sequence of SEQ ID NO. 15, or variant thereof, wherein the nucleic acid encodes a functional cyanamide resistance protein.

51. The isolated nucleic acid of claim 50, wherein the cyanamide resistance protein comprises the amino acid sequence of SEQ ID NO. 13 or variant thereof.

52. An isolated cyanamide resistance protein comprising the amino acid sequence of SEQ ID NO. 13, or variants thereof, wherein the protein confers resistance to cyanamide.

53. The isolated cyanamide resistance protein of claim 52, wherein the variant has a sequence identity of at least 80% to the amino acid sequence of SEQ ID NO. 13.

ABSTRACT

The present invention provides methods for producing transgenic plants based on an optimized transfer of DNA from *Agrobacterium* to plant cells, and/or on an optimized integration of the transferred DNAs into plant cell genomes. It also provides *Agrobacterium*-transformation vectors that can be used to limit or eliminate the transfer of undesirable DNA. The present invention can be applied to essentially any species of plants, including many recalcitrant plant species. The present invention also provides new cyanamide resistance marker genes and proteins for enhancing plant cell transformation.

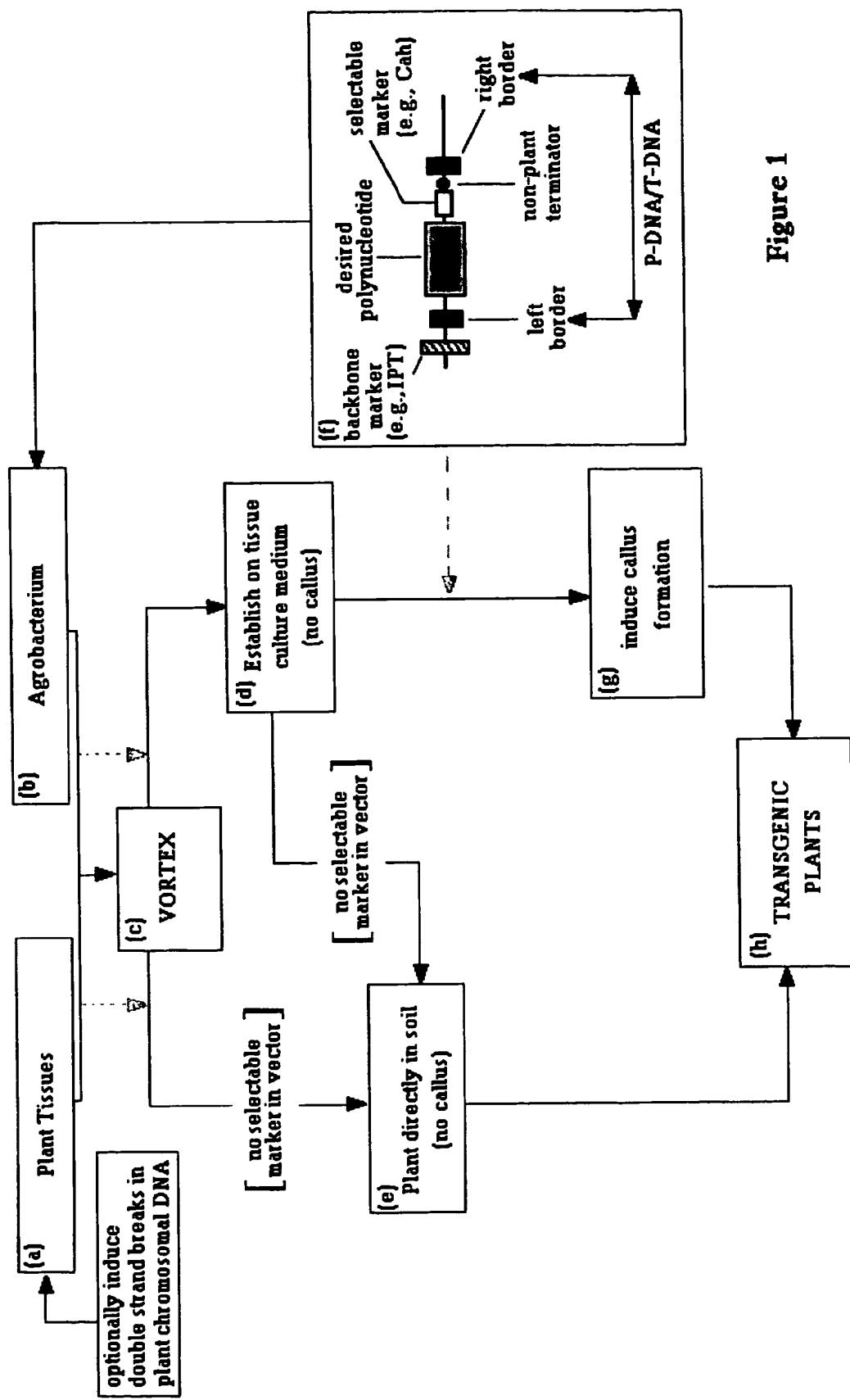


Figure 1

Figure 2

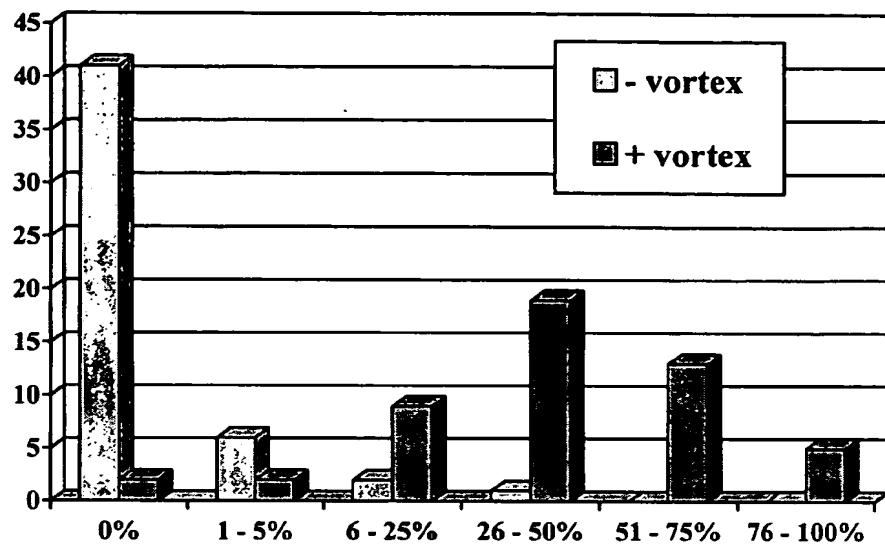
Alignment of the CAH gene from *Myrothecium verrucaria* with a new cyanamide tolerance gene isolated from *Aspergillus* (CAH-H1) and a non-functional yeast CAH homolog (CAH-H2)

CAH	MSSSEVKANGWTA	PVSAKAI	VDSL	GKLGDV	SYSVEDIAFPAA	DKLVAEAQAFVKARL	60	
CAH-H1	MCQNEVEVNGWT	SM	PDAGAI	FDGGPF	INPEAL	SIEIKFPVD	DPIVEKTMRYAKAALP	60
CAH-H2	-----MSQYGF	RVVP	REVEKA	IP-----VN	NAPRPR	AVVPPN	SETARLVREYAAKELT	49
	:.. *:: :* .. .			:..	: * :	*	.
CAH	PETYNHSMR	RVFYW-----		GTVIARRLLP	EQAKDLS	PSTWALT	CLLHD	102
CAH-H1	TETFNHSMR	VYYGMQDCASHGVL	INRSQALGMAITKQQFP	KQASALSP	STWALT	CLLHD	120	
CAH-H2	APVLNHSLRVF	QY-----	SVAIIRDQFP	--AWDLDQEVL	YVT	CLLHD	89	
	:.. ***:***: :			:.. *	: * * .	.. :	*****	
CAH	VGTAEAYFTSTRM	SFDIYGGIKAMEVLK	-VLGSSTDQAEAVAE	AAIRHEDVGVDGNITFL	161			
CAH-H1	IGTSDHNLAATRMS	FDIYGGIKALEVLK	-GFGATSDQAEAVAE	AAIRHQDLGVHG	TITYI	179		
CAH-H2	IATTDKNMRA	TKMSFEYGGILSREL	VFNATGGNQDYADAVTE	AAIRHQDLTG	GYITT	149		
	:.. *:: : :**:****: *:::			*.. * *;***:*****:*	* ** :			
CAH	GQLIQLATLYDNVG	A YDGIDDFGSWVDDTRNS	SINTAFPRHGWC	SWFACTVRKEESNK	PW	221		
CAH-H1	GQLIQLATIYDNVG	AHPYVKDFGELIHDTTR	SQVHEAHPPGEWRTFFSG	VIQKEQAIKP	W	239		
CAH-H2	GLILQIATTLDNVGS	N-----TDLIHIDTV	SAINEQFPRLHW	LSCFATVVDTENS	RKPW	203		
	* ::*:*** ****: .			* : * : * : .	:: ***:			
CAH	CHTTHIPQ-FDKQM	EANTLMKPWE	244					
CAH-H1	CHTKKMVN-VLRKG	SRRHPDGQ---	259					
CAH-H2	GHTSSLGDDFSKKV	CNTFGYN--	225					
	**. : : . : .							

Figure 3

Alignment between a new ubiquitin-like promoter (UbiN) and the corresponding part of the sugarcane Ubiquitin-4 promoter.

Figure 4. GUS-expression in *Agrobacterium*-infected alfalfa seedlings
"- vortex": subjection by immersing (30 minutes) seedlings into an *Agrobacterium* suspension; "+ vortex" subjection by vortexing (30 minutes) seedlings immersed in an *Agrobacterium* suspension.



Application Data Sheet

Application Information

Application Type:: Continuation-In-Part
Subject Matter:: Utility
Suggested classification::
Suggested Group Art Unit::
CD-ROM or CD-R?:: None
Computer Readable Form (CRF)?:: No
Title:: Refined Plant Transformation
Attorney Docket Number:: 058951-0172
Request for Early Publication?:: No
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Suggested Drawing Figure:: 1
Total Drawing Sheets:: 4
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Secrecy Order in Parent Appl.?:: No

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Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	An application claiming the benefit under 35 USC 119(e)	60/365,527	3/20/2002
This Application	An application claiming the benefit under 35 USC 119(e)	60/377,597	5/6/2002
This Application	Continuation-in-part of	10/392,301	3/20/2003

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Country::	Application number::	Filing Date::	Priority Claimed::

Assignee Information

Assignee name:: J.R. Simplot Company

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